Respiration physiology and the gill structure of the New Zealand freshwater crayfish *Paranephrops zealandicus* (White 1847) (Decapoda : Parastacidae).

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I would like to thank John Romer for the delightful history of Kenhirkhopeshef, scribe to King Ramesses II of Thebes in the 13th century B.C. Kenhirkhopeshef rose in his career to become the clerk of works, union leader, advocate and counsellor for all the builders and artists of the king's tomb. He made records of their private lives, their disputes, their work progress and the materials they used. In rare moments of frustration he would complain that the task of a scribe was not an easy one. When he died he was more than eighty years old. Building tombs and writing about it is no easier today!

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ABSTRACT

The New Zealand freshwater crayfish *P. zealandicus* emerges from water and exposes it's respiratory surface to desiccation and collapse, it's haemolymph to acid-base disturbance and it's tissues to hypoxia. This study was to investigate the respiratory system during aerial respiration.

P. zealandicus has 20 gills, a rudimentary gill and an epipodite (20 + r + ep). Two types of gill filament were identified; a respiratory filament with a cuticle 0.7 μm thick, an afferent and efferent vessel and haemolymph lacunae adjacent to the cuticle, and an ion regulating filament with a cuticle 1.2 μm thick in which the cells adjacent to the cuticle contain all the organelles and membranes which are associated with ion regulating tissues.

It was found that *P. zealandicus* emerged from the water voluntarily. There was no significant difference in the frequency of emersion by solo crayfish at 18°C, crayfish pairs at 18°C, solo crayfish experiencing hypoxia at 18°C, and solo crayfish at 24°C. Solo crayfish and crayfish pairs recorded a significantly higher level of emersion activity at night than during the daytime.

At 15°C the settled rate of oxygen consumption in air, 1.03 ± 0.03 $\mu mol.g^{-1}.h^{-1}$ (± 1 sem), was not significantly different from the settled rate of oxygen consumption in water, 1.10 ± 0.03 $\mu mol.g^{-1}.h^{-1}$. Oxygen consumption in water was compromised by declining external oxygen tension below P_{CRIT} (41 - 44 Torr). At P_{CRIT} the oxygen consumption was 1.06 ± 0.05 $\mu mol.g^{-1}.h^{-1}$. At only one time in 48 hours aerial respiration was haemolymph arterial oxygen tension below P_{CRIT}. This was after 12 hours aerial respiration, and was associated with an increase in lactate concentration to 5 $mmol.l^{-1}$, which was, however, removed during the subsequent 12 hours aerial respiration. The crayfish were not considered to be experiencing internal hypoxia for the remainder of the 48 hours in air. The total oxygen consumed during 8 hours recovering from aerial respiration was similar to the total oxygen consumed during the 8 hours settling at the beginning of the experiment, indicating no measurable oxygen debt accumulated during 48 hours aerial respiration.

After emersion into air the crayfish experienced a respiratory acidosis, and an elevated arterial carbon-dioxide tension. Total haemolymph carbonates, measured 12 hours

after emerging from water indicated compensation by metabolic alkalosis. Upon returning to the water the crayfish experienced a respiratory alkalosis. Rapidly declining arterial carbon-dioxide tension, and total haemolymph carbonates indicated compensation by metabolic acidosis. The measured *in vitro* non-bicarbonate buffer capacity was -3.8 $mmol.l^{-1}.(pH\ unit)^{-1}$. During the 48 hours in air the pH was regulated between 7.55 and 7.68 with the pH significantly more alkaline at 8 pm than at 8 am, by 0.1 pH units.

At 15°C, the solubility of oxygen in haemolymph, $\alpha_{PLASMA}O_2$, was 1.8 $\mu mol.l^{-1}.Torr^{-1}$, and at an oxygen tension of 150 Torr the total haemolymph oxygen content was 1 $mmol.l^{-1}$. There was a Bohr effect, $\Delta logP_{50}/\Delta pH$, of - 0.48 to - 0.96. Settled in water at 15°C, with a haemolymph carbon-dioxide tension of 2.6 Torr, the crayfish haemocyanin was 50 % saturated at an oxygen tension of 12 Torr. Crayfish kept at 15°C had an oxygen content when the haemocyanin was 50 % saturated, and a total oxygen content, which were 50 % higher than crayfish from water at 9.5°C.

Estimates of limitation to diffusion/perfusion, L_{diff}, in water of 0.76, and in air of 0.98, indicate that respiration in *P. zealandicus* is diffusion limited. The respiratory frequency, f_R, and heart frequency, f_H, of settled crayfish in air was not significantly different from settled crayfish in water. The f_H during aerial respiration demonstrated less fluctuation than f_H in control crayfish in water, and the f_R of crayfish in air was more variable than the f_R in control crayfish in water. The control crayfish settled in water exhibited a significant diel fluctuation in f_R and f_H, with high f_R and f_H recorded in the evening and low f_R and f_H in the morning.

Several activities and changes measured in this study have a diel rhythm. A dusk pH recorded at 8 pm. was 0.1 pH units more alkaline than a dawn pH recorded at 8 am. Settled in water, *P. zealandicus* exhibits a diel fluctuation in f_R and f_H, with high f_R and f_H recorded in the evening and low f_R and f_H in the morning. At 18°C, solitary crayfish and crayfish pairs show higher levels of emersion activity at night than during the daytime.

The results indicate that crayfish *P. zealandicus* has a respiratory system which can cope with aerial respiration, and some of the activities and changes reported in this study have a diel rhythm.

CHAPTER 1

INTRODUCTION

I. GENERAL INTRODUCTION

Written history records freshwater crayfish as far back as Aristotle (384-322 B.C.; Hart and Clark, 1989), and in the 16th century they were considered a sufficiently desirable morsel for the dining table that King John III of Sweden (1537-1592; Derry, 1979) was celebrated for importing crawfish and breeding them in his own country (Linnaeus, 1746, in Hobbs *et al., 1989*), no doubt under "royal protection". Today crayfish are part of the cuisine of many cultures, and many species are supplied by aquaculture enterprises all over the world. In a good year the southern U.S.A harvest is estimated to exceed 50 000 tonnes (Holdich and Lowery, 1988).

Freshwater crayfish belong to the order Decapoda which also contains crabs, shrimps, prawns, marine crayfish and lobsters (Table 1.1). The freshwater crayfish are divided into two superfamilies, the Astacoidea and the Parastacoidea. The Astacoidea in the northern hemisphere consist of two families, the Astacidae and the Cambaridae, with a total of 15 genera and 349 species (Hobbs, 1988). The Parastacoidea in the southern hemisphere have one family, the Parastacidae, with 129 species in 14 genera (Hobbs, 1988), and include the two New Zealand *freshwater crayfish species, Paranephrops zealandicus* and *P. planifrons* (Table 1.2).

Astacoidea and Parastacoidea are thought to have had separate marine origins, sharing a common ancient ancestor with the marine lobsters, the Nephropoidea (Hobbs, 1974a). The ancestral astacidean was very likely a widespread species in the littoral zone of temperate and subtemperate waters of the Jurassic seas 200-135 mybp (Hobbs, 1988). Even then they were probably opportunistic scavengers, and did not swim but walked. The four evolutionary lines are thought to have developed from the ancestral astacidean before the end of the Mesozoic era (Fig. 1.1). Nephropoidea have remained in the

TABLE 1.1 Classification of the order Decapoda. From Glaessner (1969) in Phillips *et al.* (1980), fossil groups not included.

Phylum – **Arthropoda**
Subphylum – **Crustacea**
Class – **Malacostraca**
Order – **Decapoda**

Suborder – Dendrobranchiata.

Infraorder	Family	Subfamily	Common names
Peneidea			Prawns
Sergestidea			Deep sea prawns

Suborder – Pleocyemata.

Stenopodidea			Cleaner shrimps
Caridea			Shrimps
Astacidea	Nephropidae	Nephropinae	Clawed lobsters
		Homarinae	
		Neophoberinae	
	Astacidae		Freshwater crayfish
	Cambaridae		
	Parastacidae		
Palinura	Glypheidae		
	Polychelidae		
	Palinuridae		Spiny lobsters
	Scyllaridae		Slipper lobsters
	Synaxidae		Coral lobsters
Anomura			Hermit crabs etc
Brachyura			True crabs

TABLE 1.2 Summary of freshwater crayfish species, ep = epipodite, r = reduced gill, epr = epipodite with gill filaments functioning as a rudimentary gill. From Hobbs (1974a) and Hobbs (1988). sp = species, ssp = subspecies.

		1988 (Counts	1974 sp:ssp)	location	gills.
Superfamily **ASTACOIDEA**					
Family ASTACIDAE.					
Genera	*Astacus*	4:4	4:6	Mid & East Europe	18+2r+ep.
	Austropotamobius	3:4	3:5	West & Mid Europe	or
	Pacifastacus	5:2	5:2	West N.America & Missouri, introduced to Sweden & Japan.	18+3r+ep.
Family CAMBARIDAE.					
Subfamily CAMBARINAE.					
Genera	*Barbicambarus*	1:0	1:0	Kentucky & Tennessee	17+ep.
	Bouchardina	1:0	----	Tennessee	17+ep.
	Cambarus	78:2	55:3	East North America	17+ep.
	Distocambarus	2:0	----	Georgia & South Carolina	17+ep.
	Fallicambarus	13:0	9:0	Central North America	17+ep.
	Faxonella	4:0	3:0	Southern North America	17+ep.
	Hobbseus	6:0	5:0	Mississippi & Alabama	17+ep.
	Orconectes	67:10	63:7	East North America introduced into West Europe	17+ep.
	Procambarus	143:10	117:6	Central North America Cuba & Guatemala.	17+ep.
	Troglocambarus	1:0	1:0	Florida	16+ep.
Subfamily CAMBARELLINAE.					
Genus	*Cambarellus*	17:0	12:0	West & South North America	16+ep.
Subfamily CAMBAROIDINAE.					
Genus	*Cambaroides*	4:3	4:3	East Asia, Korea, Japan & Amur Basin.	18+3r+ep.

TABLE 1.2 continued, ep = epipodite, r = reduced gill, epr = epipodite with gill filaments functioning as a rudimentary gill. From Hobbs (1974a) and Hobbs (1988), the genus Gramastacus was constructed by Riek in 1972 without a gill formula and Hobbs (1974a) has no further data. sp = species, ssp = subspecies.

Superfamily **PARASTACOIDEA.**		1988 (Counts	1974 sp:ssp)	location	gills.
Family PARASTACIDAE.					
Genera	*Astacoides*	6:0	1:4	Madagascar	12-13+epr+3-5r.
	Astacopsis	2:0	4:0	Tasmania	21+epr.
	Cherax	38:3	39:3	New Guinea, East & Southwest Australia	21+epr.
	Engaeus	24:0	23:0	New South Wales, Victoria & Tasmania	21+epr.
	Engaewa	3:0	3:0	Western Australia	21+epr.
	Euastacoides	3:0	2:0	Queensland	21+epr.
	Euastacus	37:11	27:0	Queensland, Victoria, New South Wales	21+epr.
	Geocharax	2:0	2:0	Victoria, Tasmania	21+epr.
	Gramastacus	2:0	2:0	Victoria	------.
	Paranephrops	2:0	2:0	New Zealand	20+epr+r.
	Parastacoides	1:2	6:0	Tasmania	17+epr.
	Parastacus	6:0	6:0	South America	20+epr+r.
	Samastacus	2:0	2:0	Chile	20+epr+r.
	Tenuibranchiurus	1:0	1:0	Queensland	18+ep, some males 20+ep.

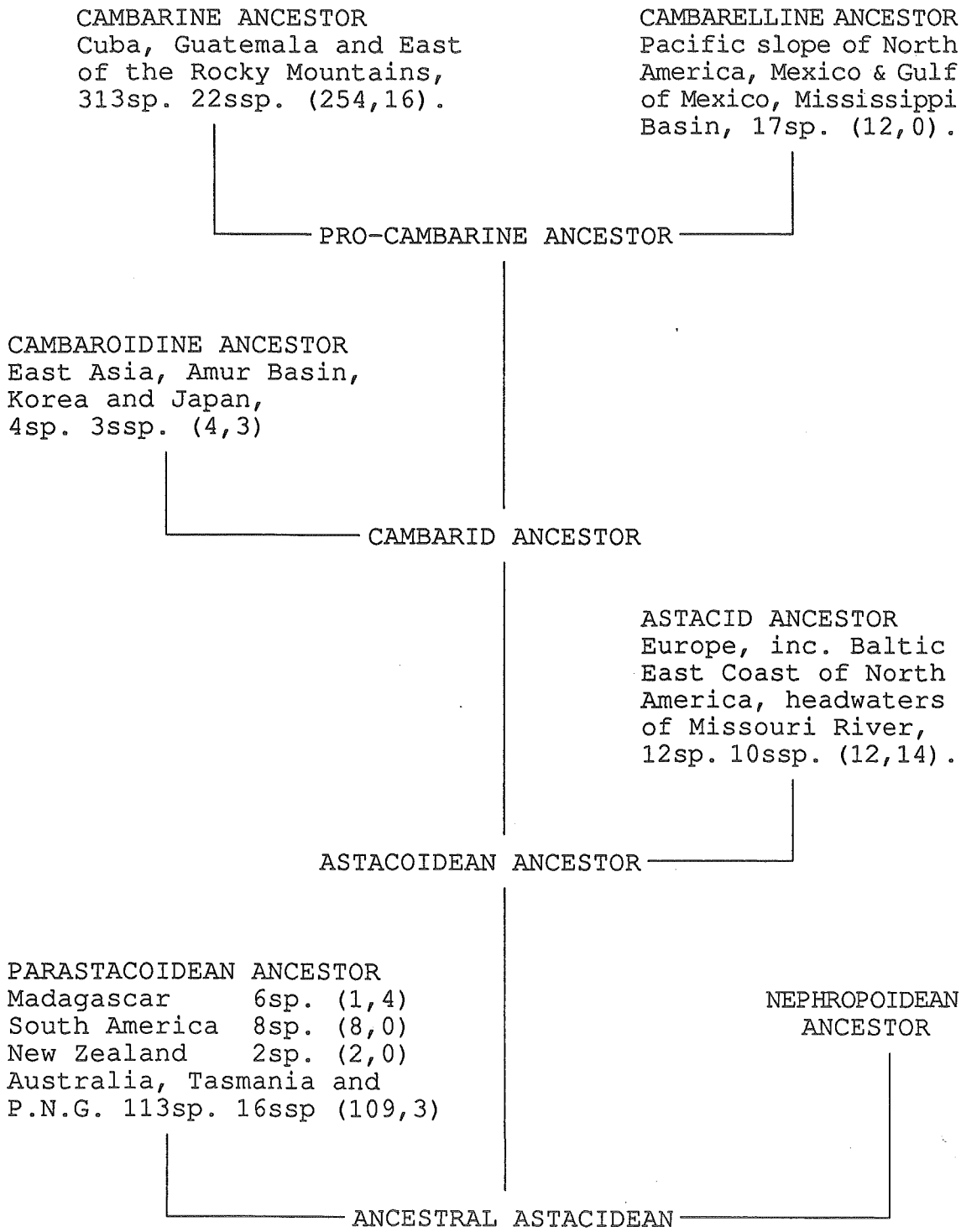


FIGURE 1.1 Phylogenetic relationships in the infraorder Astacidea, showing the distribution of the freshwater crayfish families and number of species within the families, adapted from Hobbs (1974a). The numbers are of species and subspecies from Hobbs (1988) with the 1974 values in parentheses.

sea and are generally considered the most conservative with the most plesiomorphic condition. Presumably the astacoid and parastacoid ancestors became restricted to fresh water via brackish estuarine environments (Hobbs, 1988).

Crustacea adapted to the sea require both embryological and osmoregulatory changes to successfully invade and inhabit fresh water. The eggs of marine Crustacea are in osmotic balance with the external medium and quickly hatch larvae which are also isosmotic with their environment. Survival of the eggs and larval stages in fresh water has been achieved by releasing larvae which are more developed, from fewer, larger, nearly impermeable eggs with more yolk (Vernberg and Vernberg, 1983; Hobbs, 1988). Freshwater crayfish release miniature adults which have undergone two moults to Stage III juveniles (Hopkins, 1967a).

The osmoregulatory and ionoregulatory challenges will have been similar to those faced by estuarine crabs today. Seawater has an osmolarity between 900 and 1200 $mOsm.l^{-1}$, brackish water between 15 and 900 $mOsm.l^{-1}$, and fresh water has an osmolarity below 15 $mOsm.l^{-1}$ (Rankin and Davenport, 1981). Marine animals must have gradually evolved mechanisms for hyperosmotic regulation in an intermediate brackish water environment, as the blood of freshwater animals descended from them now has a higher ion content and osmotic pressure than the surrounding external medium. The function of regulation is to provide a stable internal environment and in freshwater this means reducing to a minimum the salt losses through renal and extra-renal sites, replacing lost salts by active uptake from the external medium and food, and removing the water which is continuously drawn into the body by the osmotic gradient (Wong and Freeman, 1976c). In Crustacea this is achieved by a reduction in carapace permeability, an increase in urine production, urine hypo-osmotic to the blood, and increased activity of ion uptake systems (Rankin and Davenport, 1981).

Once the embryological and osmoregulatory changes had been made a diverse range of freshwater habitats and even some terrestrial environments became available for the crayfish to exploit.

II. PHYLOGENY AND ZOOGEOGRAPHY

Despite the diversity of morphology, there seems to be more basic uniformity among modern Parastacoidea than exists among the Astacoidea in which two families are recognised. Historically all of the information for evaluating the inter-relationships of crayfish at generic and familial levels has been derived from comparative morphology, geographical distribution and ecology (Riek, 1972; Hobbs, 1974a, 1974b, 1981). More recently immunological and electrophoretic techniques have been used to unravel the phylogeny of the Parastacoidea (Patak and Baldwin, 1984; Patak *et al.*, 1989).

The overlapping distributions of the two Astacoidean families confuses the relationship of the Cambaridae to the Astacidae, and Hobbs (1988) suggests the possibility that these two groups represent separate invasions by different marine ancestral lines. Hobbs (1988) also considers that there are fewer assumptions involved in the probability of independent invasions of the fresh waters of Laurasia and Gondwana than would be required if radiation had proceeded from a single stock. If one considers that it will never be known when, where, and how many times complete freedom from the sea was achieved by crayfish stocks during ancient times, then it is a surprise that only 3 ancient lines appear to have succeeded and survived to the present day.

(1) Astacoidea

The Astacoidean ancestor is thought to be Mesozoic or older, ie. 225 mybp or more, and the Cambarid ancestor can be considered to be older than post Jurassic, 135 mybp (Hobbs, 1988). Hobbs (1988) believes the Cambaridae to be the farthest removed from the ancestral stock for the following reasons:

1) loss of pleurobranchs in the majority of its members,
2) the unique presence of male cyclic dimorphism,
3) hooks on the ischia of some of the pereopods,
4) cornified apical prominences on the first pleopod,
5) a seminal receptacle in females, except asiatic sp.

The relationship between the asiatic Cambaroidinae and the american Cambarinae and Cambarellinae is far from clear, not just on the grounds of morphology but also in regards to dispersal, and Hobbs (1988) proposes independent invasions of Asia and Southern America by a common ancestor. All evidence points to a radiation in the Cambarinae from the south-eastern United States.

The Cambaridae are the most highly specialised of the three families of crayfish and demonstrate the greatest diversity. Within the Cambarinae there are many troglobitic species and some "primary burrowers" (see chapter 3) which are able to complete their life cycles in burrows without having to enter open water, an innovation also used by some Parastacidae. There are species in several genera which inhabit temporary bodies of water, burrowing into the substrate when the water is lost.

The family Astacidae are probably the most studied of the crayfish fauna and this has resulted in 4 taxonomic revisions in the past half century (Hobbs, 1988). Even though they appear to be the oldest of the Astacoidean groups, the Astacidae are more conservative in their use of habitats and have not colonized semi-aquatic habitats or the subterranean habitats which are abundant in Europe. Their dispersal is complex and the only agreement that seems possible is that the centre of distribution is western Asia (Hobbs, 1988).

(2) Parastacoidea

The change from the marine environment to freshwater by the pre-Parastacoidean ancestor, and the subsequent dispersal, must have occurred before the break-up of Gondwana some 200 mybp. By the close of the Cretaceous, 80 mybp, the regions representing Africa, India and Madagascar had pulled well away from the crescent made up of Australia, Antarctica and South America (Figure 1.7). Parastacoidea are now found in Tasmania, Australia, New Guinea, New Zealand, Madagascar, and southern South America with each region having a fauna endemic at the generic level. The high endemicity of the species suggests that the families of freshwater crayfish are ancient (Carpenter, 1977), but the absence of crayfish from

Africa and India raises interesting questions about dispersal and biogeography.

Parastacidae number some 129 species in 14 genera (Hobbs, 1988), and of these only 16 species are not endemic to Australia, Tasmania and New Guinea, where there are many overlapping populations (Williams, 1980). According to Riek (1972) the aggregation of the most plesiomorphic genera in the Bass Straight Basin almost certainly indicates that the monophyletic family of the Parastacidae originated from that region. In Riek's phylogeny and distribution, (Table 1.3, Figure 1.2), each of the 4 major groups has:

a) a genus in Tasmania, with 2 of the 4 Tasmanian genera also represented in continental Australia,

b) a genus outside Tasmania/Australia, in Madagascar, New Zealand or in South America (2),

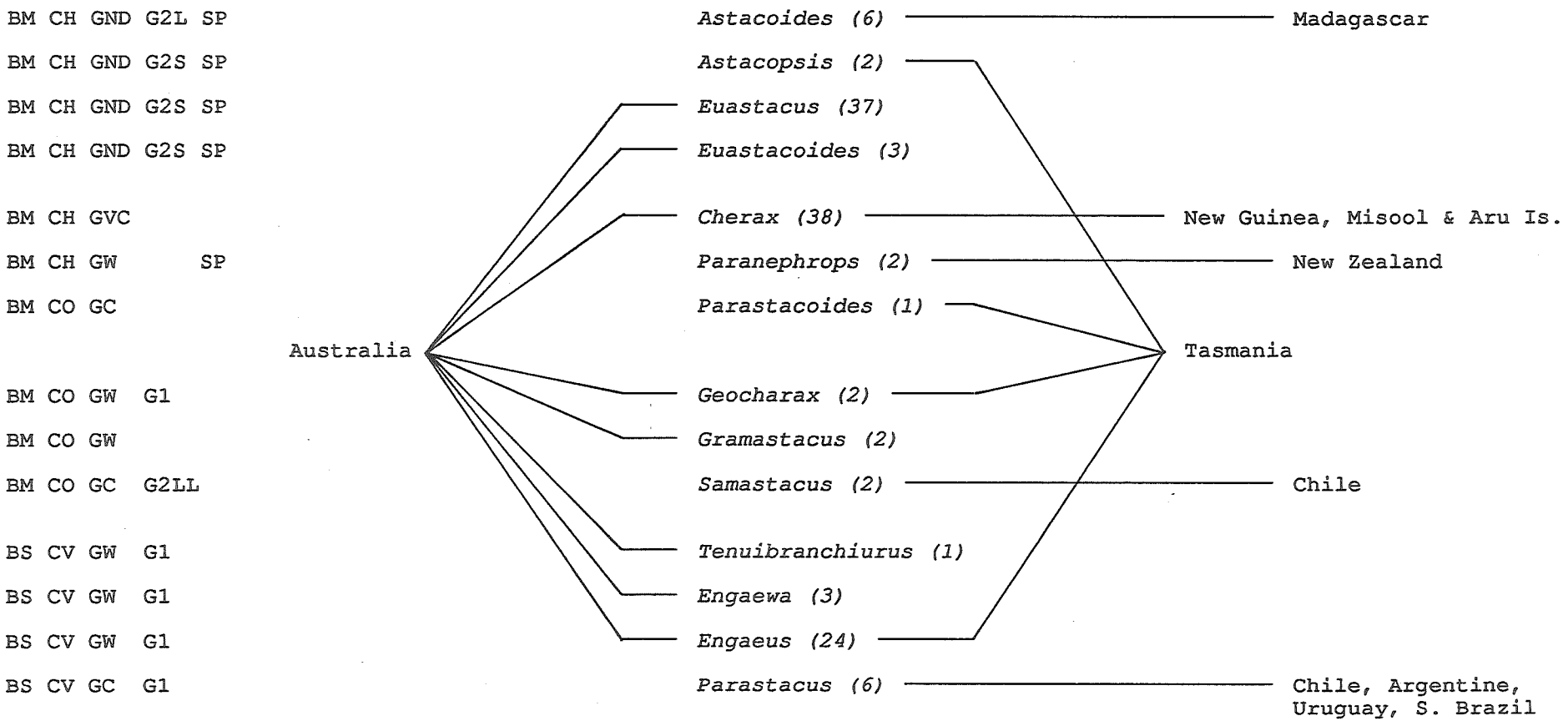
c) one or more genera in the Australian mainland.

Within each group the Tasmanian genus is the most plesiomorphic, the genus from Madagascar, New Zealand or South America is close to the Tasmanian genus, and the genera on the Australian mainland are the most apomorphic. Riek (1972) considers the group of *Geocharax, Gramastacus* and *Samastacus* to be the most plesiomorphic of the Parastacidae with *Samastacus* to be the most apomorphic of the three, and the New Zealand genus *Paranephrops* to be most closely related to the Tasmanian *Parastacoides* and the Australian *Cherax*.

Hobbs as recently as 1988 suggested that the distribution problem of *Astacoides* in Madagascar and *Paranephrops* in New Zealand cannot be claimed to be solved (Hobbs, 1988). The sharing of two rather distinct species groups by Australia and South America also suggests a divergence in the ancestral line before the separation of the continental masses, and it is not known whether or not Antarctica served as a migratory link as there is no fossil evidence to date. Huxley (1880) and Ortmann (1902) suggested that the Parastacoidea had a much wider range in the past, including Africa and India, and that the advent of fresh water crabs eliminated them from such areas as Africa. That Hobbs (1988) still mentions this problem suggests that it is not yet solved.

In New Zealand the two allopatric species of *Paranephrops*

Table 1.3 Morphological attributes of the Parastacidae, from Riek (1972): BS - strong burrower; BM - moderate burrower; CH - horizontal chelae; CO - oblique chelae, considered primitive; CV - vertical chelae, strong diggers; G1 - primitive genitalia; G2S - derived genitalia, calcareous tube short; G2L - derived genitalia, tube long; G2LL - derived genitalia, tube very long; GW - wide cervical groove; GC - close cervical groove; GVC - very close cervical groove; GND - not distinguishable cervical groove; SP - spiny crayfish. The number of species in each genus is indicated, (Hobbs, 1988).



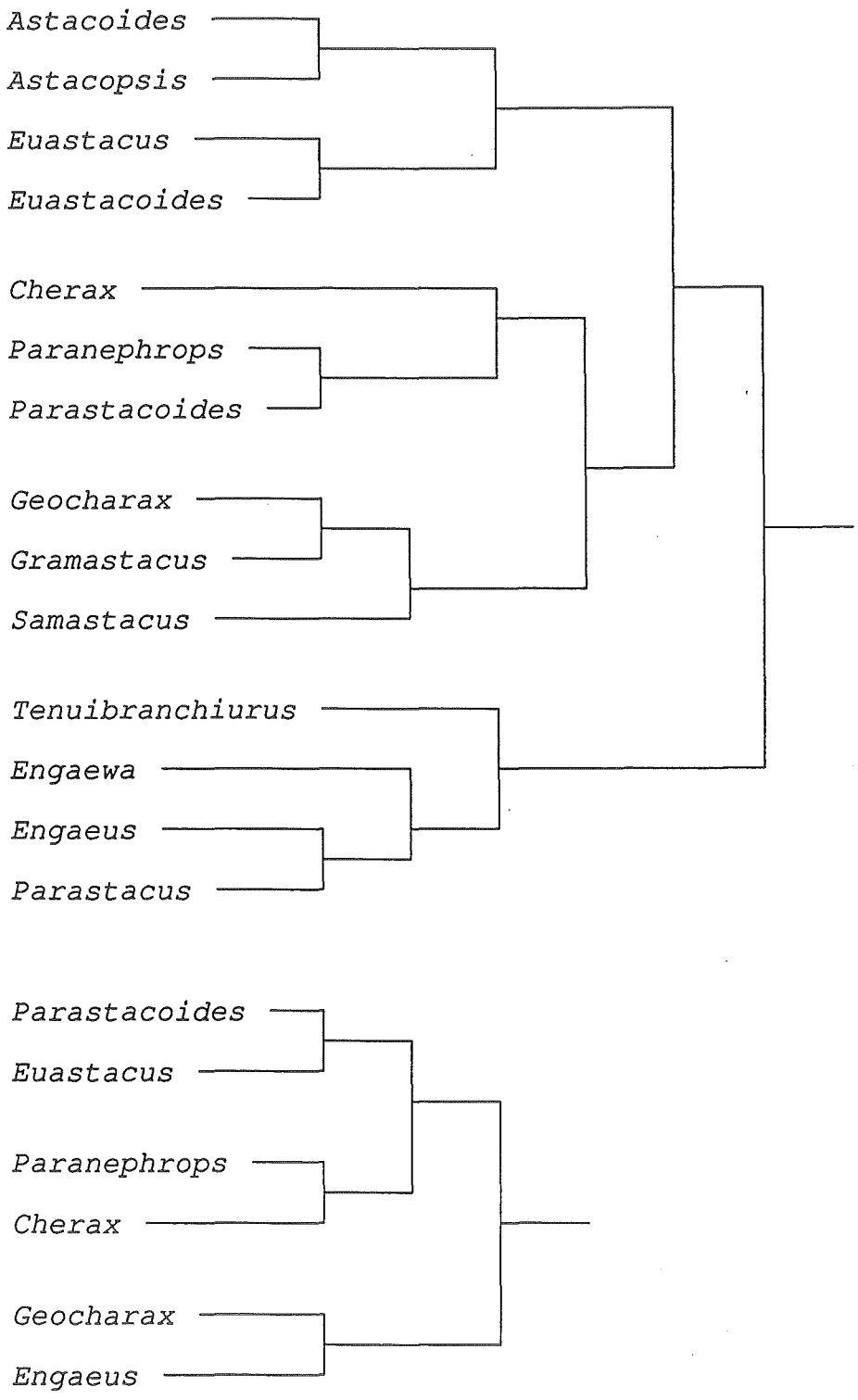


Figure 1.2 Phylogenetic relationships of the Parastacidae: The top phylogram from Riek (1972) is based on morphological differences. The bottom one from Patak *et al.* (1989) is based on immunochemical comparisons of haemocyanins. According to Riek the most apomorphic (derived) pair are *Cherax* and *Euastacus*, and the most plesiomorphic (original) are *Gramastacus* and *Geocharax* with the latter having the largest number of original features. The genus *Engaeus* is considered the land crayfish.

must have been separated at least from early Pliocene and the beginning of the Kaikoura Orogeny (13 mybp) which formed New Zealand's present geography (Fleming, 1962; Hopkins, 1970). That the distribution of other members of the native fauna has been similarly limited suggests a common upheaval (Hopkins, 1970). There were originally three species of freshwater crayfish identified in New Zealand (Chilton, 1913), but a re-examination of the systematics of *Paranephrops* by Hopkins (1970) resulted in the synonymy of *P. setosus* with *P. zealandicus*, with *P. planifrons* remaining unaltered. Both species seem to have a broad ecological tolerance and occupy similar habitats: under stones, in banks, and in aquatic vegetation in streams, rivers and lakes from sea level to an altitude between 1500m and 2500m (Carpenter, 1977), and burrowing where the bed or banks are suitable.

(3) Distribution of the Parastacoidea

According to the reconstruction of Lawver *et al.* (1992) Gondwana was still an intact mass of contiguous continents as late as the Early Jurassic, 200 mybp (Figure 1.3). If Riek (1972) is correct in his conclusion that Tasmania was the ancient source of the parastacoidean radiation then an interesting picture can be developed.

It would be reasonable to assume that the early crayfish were still entirely aquatic and had not yet ventured out of the water nor developed the associated capacity for aerial respiration. Therefore the only avenues for dispersal would be up rivers and along the coast wherever there were coastal swamps. Until they could move freely out of the water any crayfish which migrated up a river would be trapped within the catchment. There was prospect for extensive coastal migrations as the Early Jurassic boundary of Gondwana stretched from New Guinea along the East Australian coast, Northern New Zealand, Southern New Zealand, the string of Antarctic islands which terminate with the Antarctic Peninsula to Tierra del Fuego and the west coast of South America, now Chile (Figure 1.3). The genera which are represented along this coast are *Cherax* along the Australian

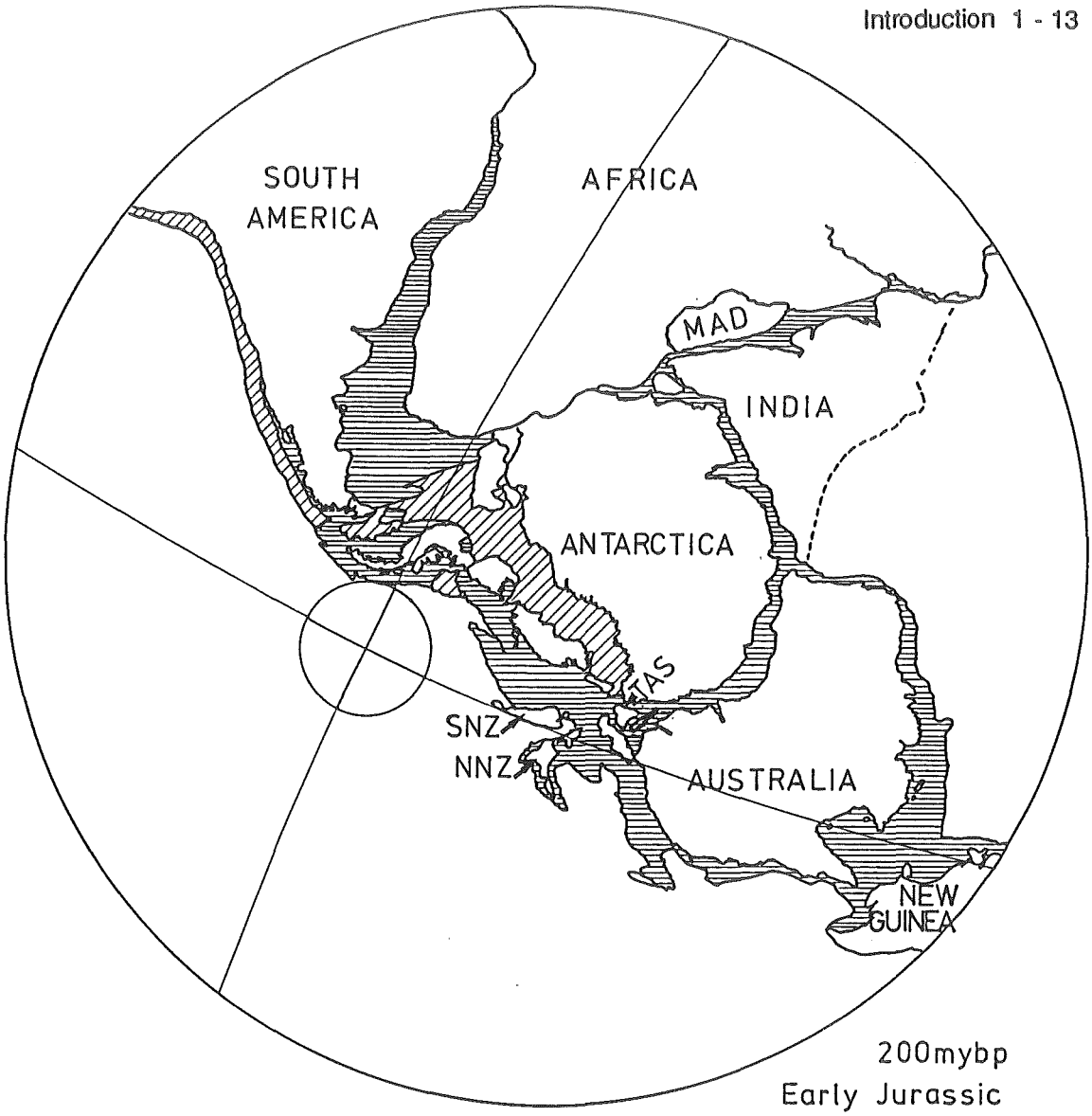


Figure 1.3 A tight fit reconstruction of Gondwana 200 mybp. The continents are indicated with coastlines and 2000-m isobaths which are shaded. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. Redrawn from Lawver *et al.* (1992).

east coast through to New Guinea, *Paranephrops* in New Zealand, *Samastacus* in Chile, and *Parastacus* in Chile, Argentine, Uruguay and South Brazil.

Riek (1972) considers *Cherax* to have the greatest number of derived characters, and the phylogenies presented by both Riek (1972) and Patak *et al.* (1989) put *Paranephrops* and *Cherax* in the same group. On the basis of morphology *Parastacus* is in Riek's (1972) out-group and *Samastacus*, a member of the most plesiomorphic group, is consequently the more likely candidate for this first coastal migration along with *Paranephrops* and *Cherax*. The dispersal of *Paranephrops* will be considered on its own later on in this section.

Figure 1.4 130 mybp Africa and South America part from India, Antarctica, Madagascar and Australia. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. From Lawver *et al.* (1992).

Three other dispersal events pose some difficulty: the dispersals of *Astacoides* to Madagascar and *Parastacus* to Chile, Argentine, Uruguay and South Brazil, and the lack of freshwater crayfish or any evidence of their past existence in Africa and India. To suggest that crayfish did arrive in Africa and India and were subsequently eliminated through competition or predation begs the question of fossil evidence. So for the moment let us take the initial position that there was no dispersal to these regions, and return to the dispersal of *Astacoides* to Madagascar and *Parastacus* to South America, and see if in trying to solve these some light may be thrown on the absence of freshwater crayfish in Africa

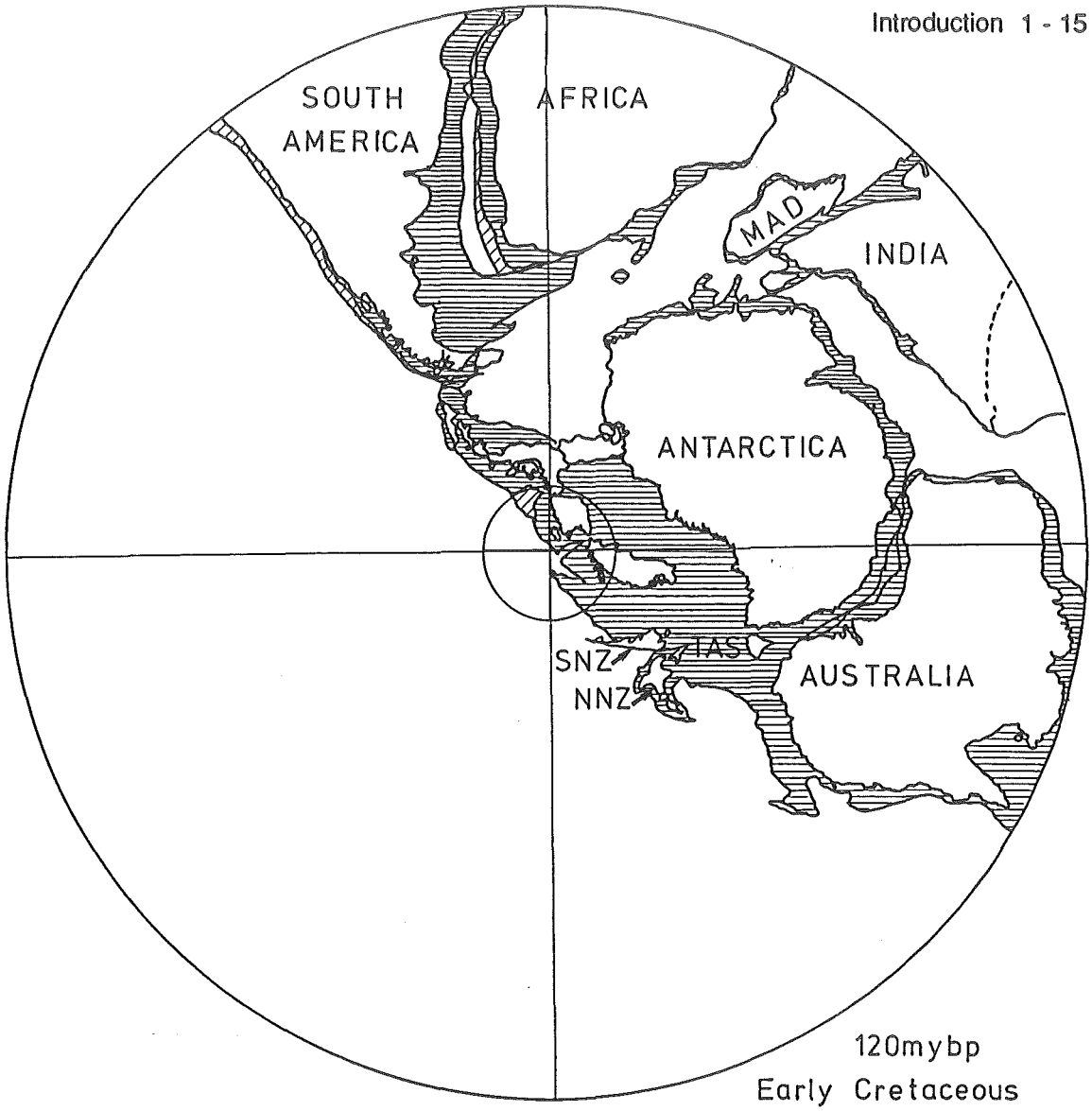


Figure 1.5 Gondwana 120 mybp, the separation of India and Madagascar from Antarctica. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. Redrawn from Lawver *et al.* (1992).

and India.

We must return to the original assumption in the discussion of dispersal, that dispersal only occurred up rivers and along coastlines. If the animal was not able to cover any distance out of water then it is difficult to consider dispersal occurring across large distances from one river catchment to another. This leaves the only viable method of dispersal as being coastal.

The phylogeny presented by Riek (1972), especially the distinctive characters described for each genus, would suggest that *Astacoides* and *Parastacus* migrated as separate stocks all the way from their ancestral source in Tasmania.

Figure 1.6 90 mybp, Africa separated from South America, Australia and Antarctica parting. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. Redrawn from Lawver *et al.* (1992).

Their migration may have been together or separated in time. The migration of *Astacoides* along a primitive coastline can only have occurred during Early Cretaceous between 130 mybp (Figure 1.4) and 120 mybp (Figure 1.5). The construction of Lawver *et al.* (1992) indicates that migration much before 130 mybp would have enabled the *Astacoidean* stock to reach Africa, and after 120 mybp the crayfish would not have been able to get to Madagascar. That Madagascar did not separate from the Indian subcontinent till some 80 mybp in the Late Cretaceous (Figure 1.7) still leaves ample time for migration to India to occur. It will take either the finding of fossil evidence to provide confirmation, and more importantly a date

Figure 1.7 80 mybp, Madagascar separated from India, New Zealand and Antarctica parting. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. Redrawn from Lawver *et al.* (1992).

for the dispersal to India, or the advancement of an appropriate reason, or comprehensive survey and lack of evidence, to indicate that dispersal did not occur.

The dispersal of *Parastacus* on the same basis also requires the availability of a coastline. As the South American and African continents were drifting apart (Figure 1.5) it is highly likely that there was, initially, a considerable fresh-water system along the line of separation. Any dispersal along this water body would have left crayfish inhabiting both continents and possibly also have resulted in a more northerly distribution. That this did not occur suggests that the crayfish dispersal did not reach the

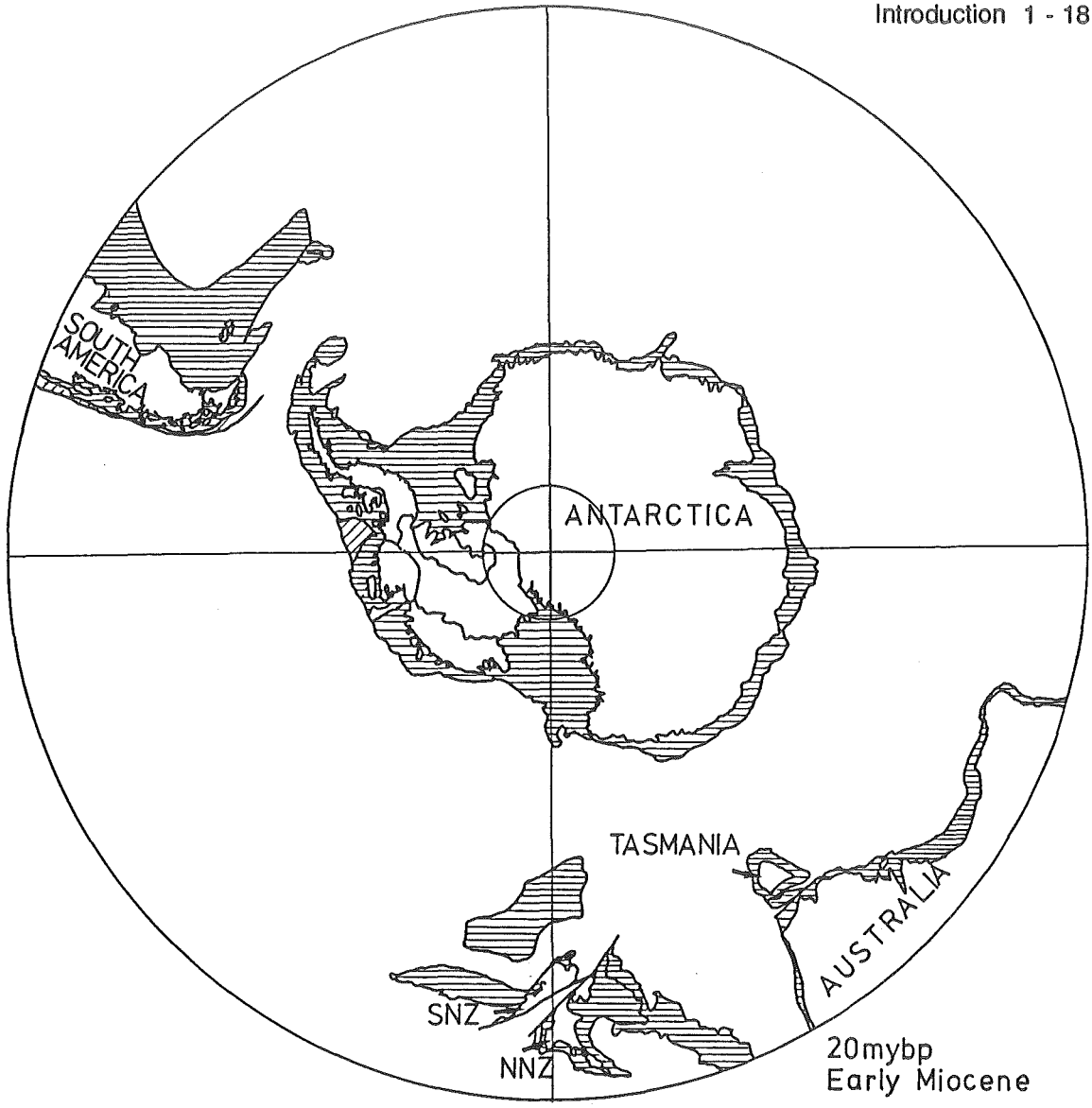


Figure 1.8 20 mybp, South America, Antarctica, Australia and New Zealand separated. MAD, Madagascar; NNZ, North New Zealand; SNZ, South New Zealand; TAS, Tasmania. Redrawn from Lawver *et al.* (1992).

eastern side of South America before the continents had separated sufficiently for the dividing water body to become saline. Thus it is suggested that *Parastacus* did not reach this region before Early Cretaceous about 120 mybp.

As can be seen it is easier to construct an hypothetical dispersal which excludes crayfish from Africa than exclusion from India, the former only requires fortuitous timing, the latter is difficult as Madagascar and India were together for at least 30 million years after the last possible arrival of *Astacoides* in Madagascar could have occurred.

Assuming the Tasmanian origin of the Parastacoidea, the migration to New Zealand of the pre-*Paranephrops* stock could

have occurred any time after the first emergence, some 200 mybp, over a period of 110 million years, up to the Late Cretaceous, some 90 mybp. Because of the proximity of New Zealand to Tasmania the more ancient time seems more probable. By the Late Cretaceous, 80 mybp (Figure 1.7), the land masses which represent New Zealand started to move away from Australia. Both Hopkins (1970) and Carpenter (1977) suggest that the two allopatric species of *Paranephrops* must have been separated at least from early Pliocene times, or some 9-12 mybp, based on a current geographical barrier, the Kaikoura-Southern Alps divide which developed during the Pliocene (Fleming, 1962). However, the map of Gondwana presented by Lawver *et al.* (1992) suggests that the two species may have been allopatric from the time of original dispersal as early as 200 mybp. The maps certainly suggest that the two species were separated from the late Cretaceous, 80 mybp (Figure 1.7), through to the early Miocene some 20 mybp (Figure 1.8).

The difficulty is that the geological history of New Zealand is not sufficiently well known for us to be certain the species have remained separated since that early time. There are many questions concerning *Paranephrops* species including, why are they so similar, and how does this reflect the historical environments to which they have been subjected? Were there previous periods of sympatry or continuous allopatry? Ultimately, is the suggestion of Hopkins (1970) and Carpenter (1977) true in a way which they could not have foreseen, that two allopatric populations or a cline were mixed and re-mixed but not separated adequately for speciation until the Pliocene and the Kaikoura Orogeny.

As stated by Hobbs (1988) " ..... too few data have been collected to make any proposed explanation of their presence or absence on these land masses entirely convincing", and this still applies to nearly all aspects of Parastacoidean distribution.

III. MORPHOLOGY AND HABITATS

Crustacea have five pairs of appendages in the head, and

members of the class Malacostraca have 14 segments in the trunk, eight in the thorax and six in the abdomen. In the order Decapoda the first three of the eight pairs of thoracic appendages are modified as maxillipeds and the remaining five pairs are legs, one pair of chelipeds and four pairs of walking legs.

The Parastacoidea differ from the Astacoidea in both the number of gills and the presence of epipodites. In the Astacoidea the epipod of the podobranchae is plate-like and that of the first maxilliped is devoid of branchial filaments. In the Parastacoidea there is no epipodal plate on the podobranchae and the epipod of the first maxilliped bears a number of well-developed branchial filaments (Hobbs, 1988; Holdich & Reeve, 1988). Also, in the Astacoidea the first pair of pleopods in the male is modified for sperm transfer and in the male Parastacoidea pleopod 1 is vestigial and in the females it is absent (Holdich and Reeve, 1988). Young Astacoidea hatch as miniatures of the adult and are attached to the mother by a telson thread. The young Parastacoidea are not attached to the mother but cling to her instead with their pereopods (Hobbs, 1988). In all crayfish the female genital aperture is located on the 6th thoracic segment and the male genital papillae are on the 8th thoracic segment (Hobbs, 1988; Figures 1.12 and 1.13).

All Astacidae are epigean, and are confined to streams and lakes with their excavation limited to burrows in the beds and banks of streams and lakes. Generally they exhibit a broad ecological tolerance with *Astacus pachypus* and *A.leptodactylus* able to thrive in brackish water (Hobbs, 1988). Astacidae do not generally emerge from the water, but have been known to forage near the water's edge at night when it is cool and moist (Brinck, 1983).

The Cambaridae are more diverse in their habit, with a number of species being hypogean, some having been in caves long enough to have lost their carapace pigment and eyes. Many species have terrestrial burrows which have water or mud in the bottom and the crayfish can be found in a state of torpor or aestivation in a moist burrow during a dry spell in the climate, or during the winter season, only emerging under

conditions of hypoxia (Hobbs, 1974b, 1981; Hogger, 1988). *Procambarus clarkii*, a burrowing species, can survive for several months in a moist burrow with low oxygen levels, and many other species can live out of water for long periods provided the air in the gill chamber is moist and they can extract enough oxygen to survive (Holdich and Reeve, 1988).

The Parastacidae, as adults, vary considerably in size from species only a few centimetres in length to the giant *Astacopsis gouldi* of Tasmania which can weigh up to 4.5 kg, and is the largest known freshwater invertebrate (Hobbs, 1988). Many of the Parastacidae can be described as semi-aquatic and even terrestrial, not because they are independent of water but because their burrows are often distant from water bodies. Some species of *Cherax* are equally at home in water or in burrows in dry or moist soil and species of *Engaewa* dig complex burrows in swampy areas along the margins of streams (Hogger, 1988). Among the species of *Engaeus* are some which burrow some distance from standing water and live in communal burrows. The population in such a burrow is believed to consist of two or more broods belonging to a single family. Some *Engaeus* are more highly modified for burrowing than the burrowers of any other group (Hobbs, 1988). In Australia many species of *Engaeus* and *Cherax* construct burrows which never contact the water table and only contain water derived from occasional surface run-off. These burrows are not found in situations where they can be flooded and the crayfish within the burrows are active at all times of the year and never appear in open water (Horwitz and Richardson, 1986; Hogger, 1988).

There appears to be a difference between the Astacidae and Parastacidae in their propensity to "get up and walk - out of the water". That crayfish in Australia have been found in artificial ponds miles from the nearest population (Hogger, 1988) suggests they must be able to withstand the physiological stresses of aerial respiration and desiccation involved in such a migration. This must also be true for those species which build their burrows some distance from water.

IV. *PARANEPHROPS* - THE NEW ZEALAND CRAYFISH

There were three species of freshwater crayfish recorded in New Zealand by Chilton in 1913, *Paranephrops planifrons* White 1842, *P. zealandicus* (White 1847) and *P. setosus* (Hutton 1873). In a review of *Paranephrops* systematics, Hopkins (1970) synonymised *P. setosus* with *P. zealandicus*. Musgrove (1988a) suggests that speciation may be incomplete as fertile offspring have been reported from cross-breeding between *P. zealandicus* and *P. planifrons*. The characters used by Hopkins (1970) to separate the two species are

- the antennae of *P. zealandicus* can reach no further back than the third abdominal segment and the propod of the cheliped is very hairy (Figure 1.9),
- the antennae of *P. planifrons* reach further back than the fourth abdominal segment and the propod of the cheliped is smooth except for a few bristles.

P. planifrons occurs throughout the North Island and in the Marlborough, Nelson and West Coast districts of the South Island. *P. zealandicus* is only found on the east coast of the South Island from North Canterbury all the way south including Steward Island, and east of the Southern Alps, (Hopkins, 1970). Both species occur in a wide range of habitats: lakes, ponds, streams and swamps, and on gravel, rocky, vegetated or muddy substrates (Hopkins, 1970; Chapman and Lewis, 1976; Carpenter, 1977). *Paranephrops* shelters in dense aquatic vegetation, under large boulders and logs in streams or lakes, and digs deep burrows in the clay banks of a lake or stream where the substrate permits, especially if there is a strong current (Hopkins, 1970). The burrows are not always simple blind holes but may link with a passage parallel to the stream, and some rise till the end is out of the water. The need for burrows and shelter was probably associated with daytime predators such as the kingfisher, *Halcyon sanctus*, and the shag, *Phalacrocorax carbo*, (Scott and Duncan, 1967; Devcich, 1979). In a more recent study, however, preliminary analysis of the gut contents of stoats, *Mustela ermina*, from South Westland, found that one in three contained remains of small crayfish, which in this location

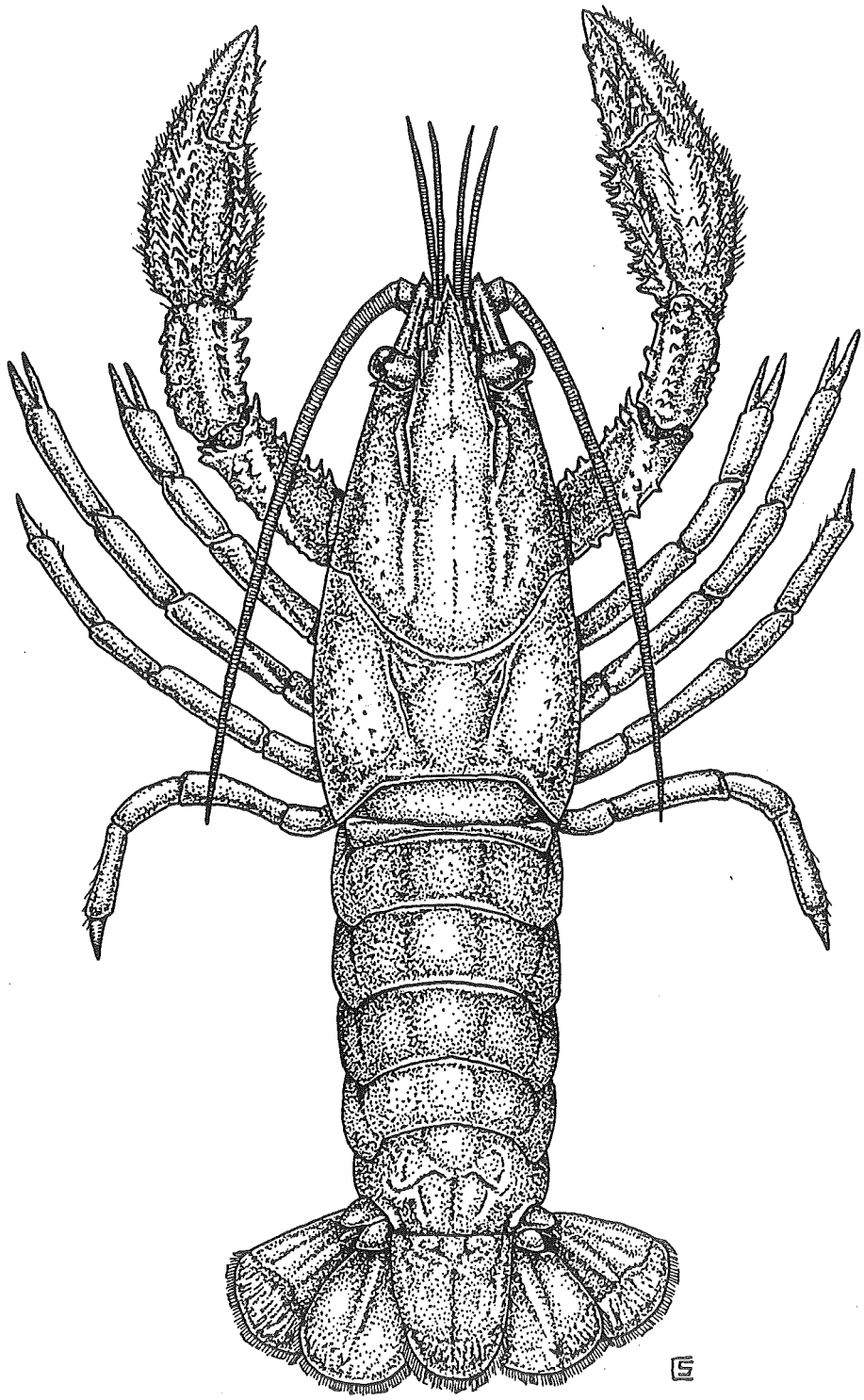
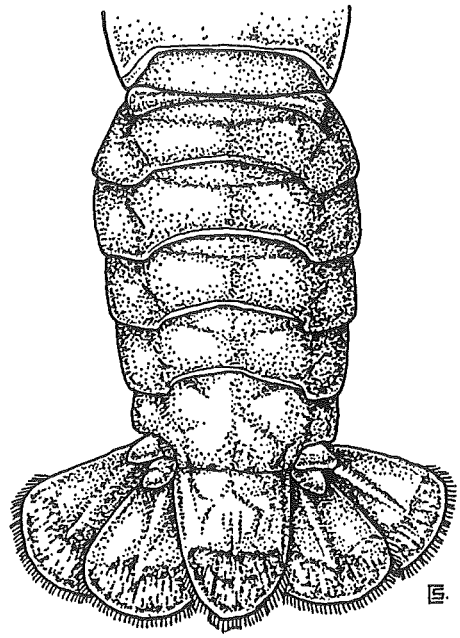
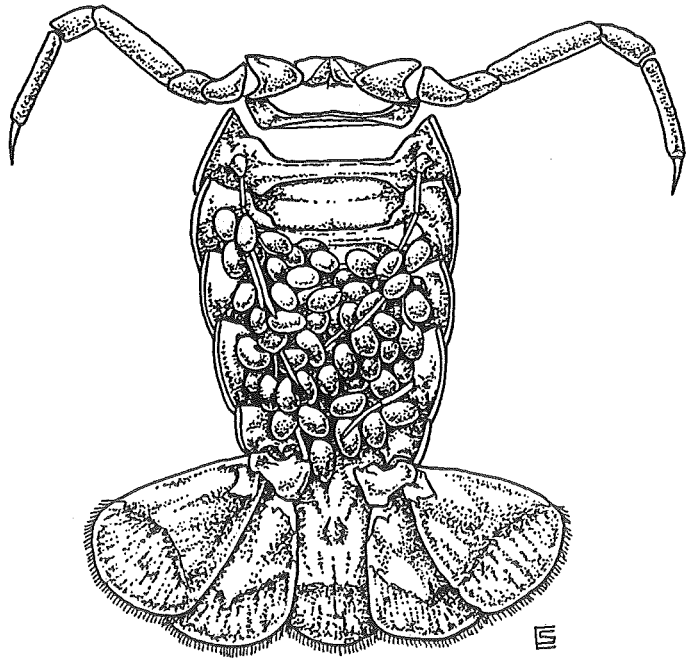


Figure 1.9 The antennae of *Paranephrops zealandicus* reach no further back than the third abdominal segment and the chelae are hairy and have numerous spines and tubercles like the carapace. This is a male.



1 cm

Figure 1.10 Dorsal view of the abdomen of a female *P. zealandicus*. The lateral margins of the abdomen are flattened and wider than in the male to provide a larger surface to protect the eggs underneath.



1 cm

Figure 1.11 Ventral view of the abdomen of a female *P. zealandicus* with eggs, a condition sometimes described as "in berry". A large female crayfish may carry up to 150 eggs attached to the setose pleopods.

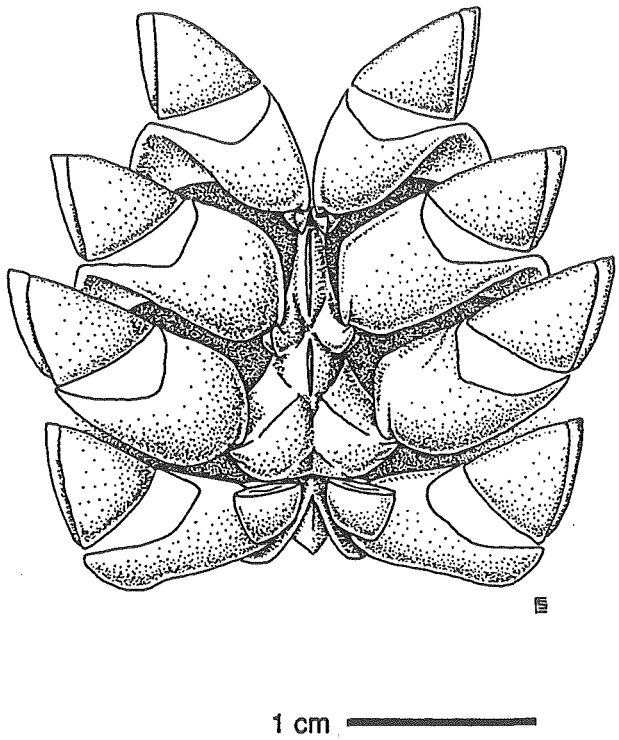


Figure 1.12 A view of the sternum of a male *P. zealandicus* with the genital papillae at the base of the fourth pair of walking legs, which are on the 8th thoracic segment.

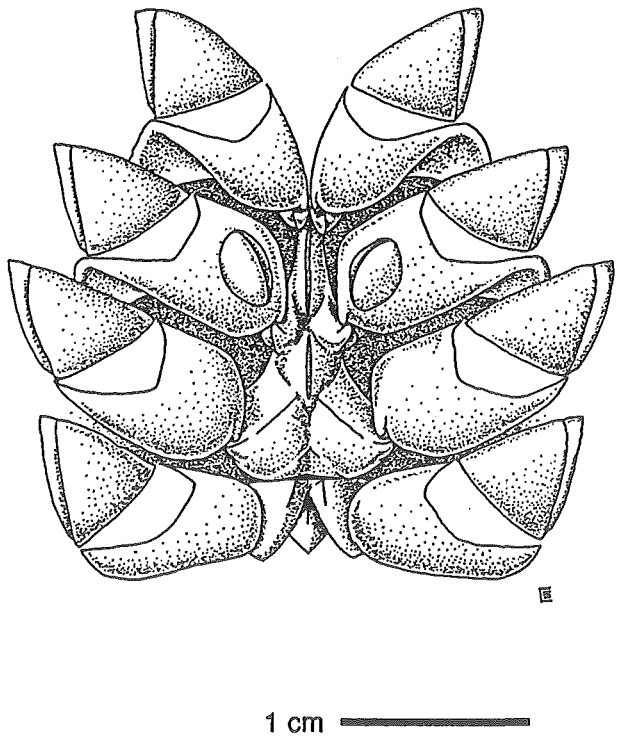


Figure 1.13 A view of the sternum of a female *P. zealandicus* with the genital openings at the base of the second pair of walking legs, which are on the 6th thoracic segment.

is *P. planifrons* (C. Rickard, *pers comm.*).

Crayfish are omnivorous bottom feeders, eating fresh and degraded detritus, live aquatic macrophytes, invertebrates and small fish, both dead and freshly caught (Chapman and Lewis, 1976; Devcich, 1979; Musgrove, 1988a).

The sexes can be most easily distinguished by the genital papillae at the base of the last pair of walking legs of the male (thoracic segment 8, Figure 1.12), and the genital pore or opening, at the base of the second pair of walking legs of the female (thoracic segment 6, Figure 1.13). From a dorsal view, the mature female will also have a broader abdomen (Figure 1.10) than the male (Figure 1.9).

Mating occurs shortly after a female has moulted and involves the male depositing a gelatinous mass containing sperm to the underside of the cephalothorax of the female. The spermatophore, which is up to 12 mm × 8 mm, is at first soft and semi-transparent, but turns slightly off-white and firms within 48 hours. It is deposited on the sternal plate of the female at the base of the last 2 to 3 pair of walking legs (Devcich, 1979). Males and non-breeding females have few setae on their pleopods, but breeding females have numerous setae on the margins of the pleopods. A female with a 30 mm carapace length can produce up to 150 eggs, and when these are fertilized, they are attached under the female abdomen (Figure 1.11) to the numerous setae on the pleopods (Hopkins, 1967a; Devcich, 1979).

Although breeding appears to be continuous as females can be found with eggs throughout the year, Devcich (1979) found that *P. planifrons* had egg laying peaks from April to July and from October to January. In May to July some 40% to 50% of mature females carried eggs, and incubation over winter took about 28 weeks while incubation over summer took only 19 to 20 weeks (Devcich, 1979). Devcich (1979) found that sexual maturity occurred in males greater than 27 mm and females greater than 31 mm carapace length, though Hopkins (1967a) mentions an ovigerous female *P. planifrons* of 17 mm carapace length. The largest sexually competent crayfish found by Devcich (1979) were a female of 70.9 mm, and male of 63.2 mm carapace length.

When they hatch the juvenile crayfish cling to the pleopods with strongly bent claws on the last two pairs of their walking limbs, and they remain attached to the abdomen of the female until their third moult when they finally release. In lakes the females carrying mature eggs and hatchlings migrate to the littoral zone where the young are released (Devcich, 1979). Devcich (1979) shows that at 20°C there is a greater growth rate than at 10°C, and that there is a survival advantage in the juveniles remaining in the littoral zone. Provided there is sufficient cover and food the juvenile crayfish come to reproductive maturity more quickly in the warmer shallow water.

Hopkins (1966, 1967a, 1967b) in his studies of stream crayfish estimated that few live longer than four years, or achieved a carapace length greater than 32 mm. However he reports a carapace length of 72.1 mm for *P. planifrons* and 80.0 mm for *P. zealandicus* (Hopkins, 1970). Devcich (1979) found a female *P. planifrons* with a carapace length of 70.9 mm, and he considers that large members of a lake population may be 11 to 16 years old and possibly up to 20 years old. In the lake population which he studied 50% of females survived six years and 50% of males survived seven years, and less than 10% of females survived 9 years and less than 10% of the males survived for 10 years. These crayfish are much older and larger than the stream crayfish studied by Hopkins (1966, 1967a, 1967b). The largest *P. zealandicus* encountered during the current study was a blue specimen weighing 180 g, with a total length of 168 mm and an OCL of 64 mm, collected from a man-made lake near Alexandra, Central Otago. The OCL measurement is from the posterior border of the eye socket to the mid-dorsal posterior border of the carapace (Hopkins, 1967a), not including the rostrum as in Hopkins (1966).

The colours of crayfish vary considerably and reflect the chemistry of the water, the colour of the substrate and the genetics of the population. They may be a very red-brown in iron-rich waters of the West Coast streams, brown to almost black, mottled green and mottled light to dark blue. There are no known crayfish plague diseases in New Zealand, but there are two commensal Turbellaria, *Temnocephala novae-*

zealandiae which is very common and lives externally about the chelae, mouth parts and base of the legs, and *Didymorchis paranephropis* which is found on the gills (Chapman and Lewis, 1976).

Wong and Freeman (1976a) suggest that *Paranephrops* species have substantially higher sodium and chloride ion concentrations and total osmotic pressures than northern hemisphere crayfish. They found that in *P. planifrons* the haemolymph osmotic pressure is between 460 and 500 $mOsm.l^{-1}$, and in *P. zealandicus* between 490 and 530 $mOsm.l^{-1}$. The ionic concentration of *Paranephrops* haemolymph varies seasonally with temperature, concentrating as the temperature drops, (Wong and Freeman, 1976b), but is independent of the external medium concentration (Wong and Freeman, 1976a). Greenaway and Lawson (1982) studied three Australian freshwater crayfish, *Cherax destructor* (in the same phylogenetic group as *Paranephrops*, Figure 1.2), *Euastacus spinifer* and *E. keirensis*, and found their haemolymph [Na], [K], [Ca], [Mg] and [Cl] to be within the range reported for Northern Hemisphere crayfish. They consider the three crayfish to be less well adapted for sodium regulation in freshwater, with low sodium affinity and a compensating high rate of sodium transport, compared with well adjusted freshwater animals. Ball (1987) in his study of the two *Paranephrops* species found that they were remarkably acid tolerant, up to pH 4, in contrast to the great majority of the Astacoidea. In a study of the blood glucose levels of *P. zealandicus*, Quilter (1977) found that removing the eyestalk caused hypoglycaemia by ending the supply of hyperglycaemic hormone which is produced in the eyestalk, and severing optic nerves removed a pathway inhibiting hyperglycaemic hormone, producing hyperglycaemia.

P. planifrons in Lake Rotoiti demonstrate a high degree of diel mobility rhythm (Devcich, 1979). Below 20 m depth the crayfish remained unconcealed and fed continually, but in shallower water the crayfish remained in cover during the day and fed only at night. At dusk there was a general migration to feed in shallower water and at dawn the crayfish returned to the deeper parts of the lake. Devcich (1979) considered that the nocturnalism reduced the losses to visual predators,

both birds and fish. *P. zealandicus* also displayed a diel activity pattern under natural conditions, with an activity peak early to middle of the night, which may be bimodal with another peak at dawn (Quilter, 1975). The level of activity increased as the temperature increased, and great individual variation in activity levels was observed. Diel rhythms in locomotor activity, metabolic rate and haemolymph physiology have been observed in many Astacoidea (Fingerman and Lago, 1957; Rice and Armitage, 1974; Pollard and Larimer, 1977; Rutledge and Pritchard, 1981; Abrahamsson, 1983; Capelli and Hamilton, 1984; Massabuau *et al.*, 1984; Sakakibara *et al.*, 1987) and crabs (Palmer, 1971).

V. THIS STUDY

For historical reasons the northern hemisphere crayfish have been studied extensively and much is known about their physiology, population dynamics, ecology, reproduction and general biology. The same cannot be said of the Parastacidae in the southern hemisphere. While the Parastacidae make up 129 (27%) of the approximately 480 species of freshwater crayfish, they only feature in about 7% to 9% of the total literature (Hart and Clark, 1987), with perhaps even more bias in the physiological literature.

Many species of *Cherax, Engaeus, Geocharax* and *Parastacoides* have terrestrial habits, displaying an ability and tendency to move about on land away from water. This suggests that the Parastacoidean morphology and physiology needs to be investigated and compared with that of the Astacoidea. There is considerable anecdotal evidence that both *Paranephrops* species have been found wandering in open beech forest and brush some distance from free water (Hillary, 1989; K.W. Duncan, *pers. comm.*).

The work by Dunel-Erb *et al.* (1982) has attributed an ionic regulatory function to the epipodite-type laminae on the podobranchae and this raises an obvious question - if the laminae are associated with ionic regulation then where and how is this achieved in the Parastacidae, especially as there is evidence of very active mechanisms for regulating the

ionic status of these freshwater crayfish (Wong and Freeman, 1976c; Greenaway and Lawson, 1982).

In view of the differences in morphology, chemistry and behaviour, especially a tendency by many species to leave the water and expose their respiratory surfaces to the high risk of desiccation and collapse, a study of the New Zealand freshwater crayfish to determine their capacity to function in air as well as in water is of considerable interest.

This study is to develop a better understanding of the respiratory system of *P. zealandicus*, a species found in Canterbury, Otago and Southland (Carpenter, 1977), using animals collected from the stream flowing into Lake Georgina (40°18'S, 171°34'E) west of Christchurch city. There will be a brief look at the gross morphology, number, location and orientation of the gills of *P. zealandicus* in Chapter 2. Does *P. zealandicus* leave the water voluntarily, and are there environmental pressures which may influence emersion from the water, are questions which will be investigated in Chapter 3. In Chapter 4 settled animals are used to establish the oxygen consumption in water and air. Resting oxygen consumption in air is important, for if the crayfish cannot maintain adequate oxygen consumption in air then emersion can only be used for brief excursions and cannot provide a refuge from environmental pressures.

Chapter 5 investigates changes to crayfish haemolymph while the animal is out of the water and forced to use aerial respiration for 48 hours. Commonly when an aquatic animal leaves the water and commences aerial respiration there are considerable changes in the haemolymph oxygen and carbon-dioxide content, pH, and total carbonate buffers. Also, in response to stress elevating oxygen demand or temporary impairment of respiration reducing oxygen supply, Crustacea usually experience a large, but often temporary, increase in L-lactate concentration in their haemolymph, produced by anaerobic metabolism.

Measuring the effects of a declining oxygen tension, in the water, on crayfish oxygen consumption, to determine when resting oxygen consumption becomes compromised, is discussed

in Chapter 6. Oxygen solubility in haemolymph, acclimation temperature, pH and carbon-dioxide content, all affect the oxygen affinity and oxygen carrying capacity. These factors are investigated in Chapter 7 to discover how much oxygen is carried by the haemolymph of *P. zealandicus*, and how well the haemolymph pH is maintained or controlled in the face of the changes brought about by aerial respiration. In Chapter 8 the heart and scaphognathite rates of the crayfish, in water and in air, are discussed.

In the final chapter this information is assembled and discussed to determine if the New Zealand freshwater crayfish *P. zealandicus* has a respiratory system which facilitates and supports some degree of aerial respiration, and if it is possible to consider the crayfish to be a facultative air-breather. By comparing the changes in oxygen consumption and haemolymph which occur in the move from water to air and back again with the changes experienced by other Crustacea, it may be possible to determine if adaptive changes have occurred which enable *P. zealandicus* to accommodate the physiological changes which are normally associated with a move from aquatic to aerial respiration. Temporal changes in activity, oxygen consumption, haemolymph, respiration and ventilation which become evident during the study will be discussed in terms of the observed natural behaviour of *P. zealandicus* and rhythms reported for other Crustacea.

V. ABBREVIATIONS

The abbreviations and symbols used in this thesis are given below.

Abbreviation	Description	Units
$\alpha_{PLASMA}O_2$	solubility of O_2 in plasma	µmol/l/Torr
$ß_{DW}O_2$	capacitance of distilled water for O_2	µmol/l/Torr
$ß_{SW}O_2$	capacitance of sea water for O_2	µmol/l/Torr
CO_2	total oxygen content	$\mu mol.l^{-1}$
CCO_2	total CO_2 content	$\mu mol.l^{-1}$
f_R	respiration frequency	$beats.min^{-1}$
f_H	heart frequency	$beats.min^{-1}$
$[HCO_3^- + CO_3^{-2}]$	total carbonate concentration	$meq.l^{-1}$
[lactate]	lactate concentration	$mmol.l^{-1}$ *
$\dot{M}O_2$	oxygen consumption	$\mu mol.gm^{-1}.hr^{-1}$
P_{50}	the PO_2 at which H_{cy} is 50 % saturated	Torr, (or mm Hg)
P_{Branch}	branchial chamber pressure	cm H_2O
P_{CRIT}	critical PO_2 at which oxygen consumption is compromised	Torr, (or mm Hg)
PCO_2	CO_2 partial pressure	Torr, (or mm Hg)
P_aO_2	arterial O_2 partial pressure	Torr, (or mm Hg)
P_vO_2	venous O_2 partial pressure	Torr, (or mm Hg)
PO_2	O_2 partial pressure	Torr, (or mm Hg)
P_IO_2	inspired O_2 partial pressure	Torr, (or mm Hg)
P_EO_2	exhaled O_2 partial pressure	Torr, (or mm Hg)
$\dot{V}_w$	ventilation volume	$l.gm^{-1}.hr^{-1}$
$\dot{V}_w/\dot{M}O_2$	ventilatory requirement	$ml.\mu mol^{-1}$

* Note - concentrations of ions are written [.....] and expressed as mol/l, mmol/l or µmol/l, ($mol.l^{-1}$, $mmol.l^{-1}$ and $\mu mol.l^{-1}$).

CHAPTER 2

GILL MORPHOLOGY

I. INTRODUCTION

1) General introduction

Crustacean appendages are typically biramous and consist of an inner part called an endopodite (or endopod) and an outer part called an exopodite (or exopod). They have five pairs of appendages in the head, a pair of antennules and a pair of antennae, a pair of mandibles which have grinding and biting surfaces, and two pair of maxillae which are accessory feeding appendages (Barnes, 1980). The class Malacostraca has 14 segments in the trunk, eight in the thorax and six in the abdomen. In the order Decapoda the first three of the eight pairs of thoracic appendages are modified as maxillipeds and the remaining five pairs are legs, one pair of chelipeds and four pairs of walking legs (Figure 2.1).

The head and thoracic segments are fused together dorsally and the sides of the overhanging carapace (called branchiostegites) enclose the gills in well defined lateral branchial (gill) chambers (Figure 2.2). The water flow within the branchial chamber is through openings at the ventral edge of the branchiostegite by the bases of the walking legs, over the gills and out via an anterior opening where there is a pump called the scaphognathite. The scaphognathite is the exopodite of the second maxilla (Grove and Newell, 1981), (Figure 2.1). Crayfish gills are trichobranchiate, with filaments about the gill shaft, and in some species there may be an associated lamina, called an epipodite, present on some of the gill shafts.

The gills are of 3 types and their names reflect the points of attachment (Figure 2.2). The pleurobranchae are attached to the pleural (lateral) wall of the branchial chamber. The coxa is the first segment of the thoracic limb and the anterior and posterior arthrobranchae are attached to the flexible arthrodial membrane between the coxa and the

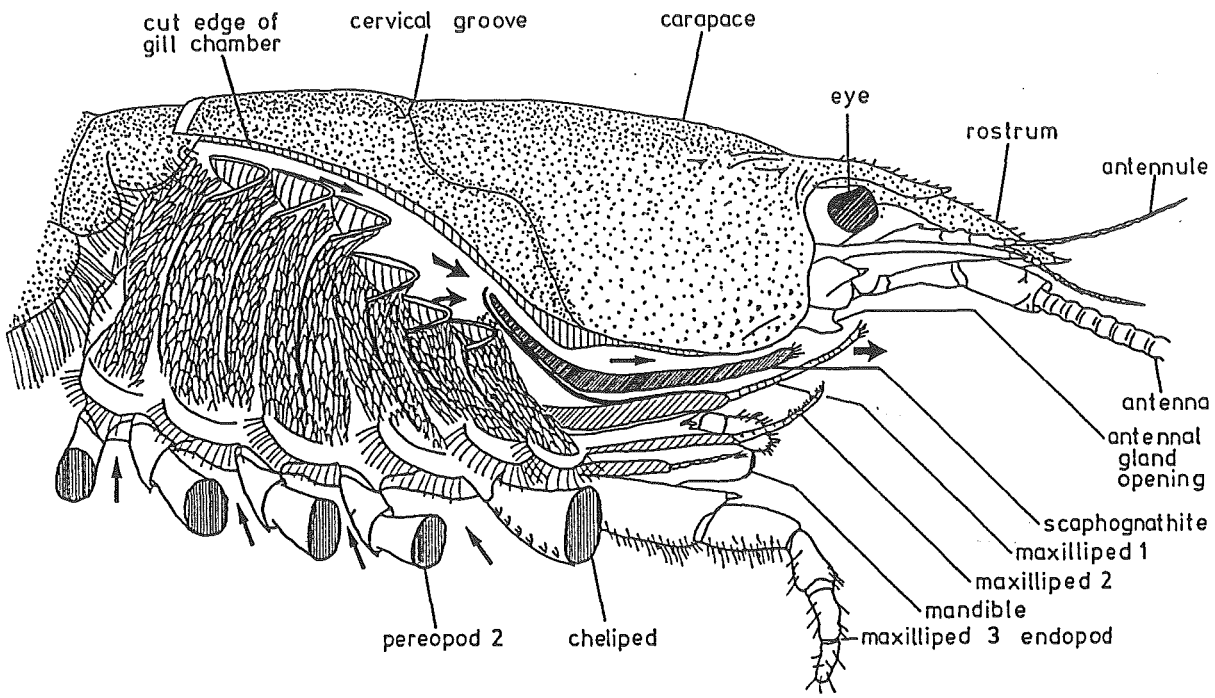


Figure 2.1 View of the branchial chamber of *Pacifastacus leniusculus* with branchiostegite removed to show the gills, limbs and direction of the ventilatory current. Redrawn from Holdich and Reeve (1988).

body wall. The podobranchae are attached to the coxae or first segment of the maxillipeds, cheliped and walking legs. Theoretically there can be four gills on each side of the eight thoracic segments, or potentially 32 gills on each side, but this condition is never found.

2) Astacoidea

In the Astacoidea there is a lamina, called an epipodite, present on some of the gill shafts. These are membranous structures which may function to keep the gills separate, regulate the branchial water flow, or both. These structures may be the distal quarter of the podobranch, as in *Procambarus clarkii* (Burggren *et al.*, 1974), an expanded epipodite, as in *Jasus novaehollandiae* (Rogers, 1982), or the podobranch may divide to produce both a gill plume and a broad thin lamina bent upon itself longitudinally, as in

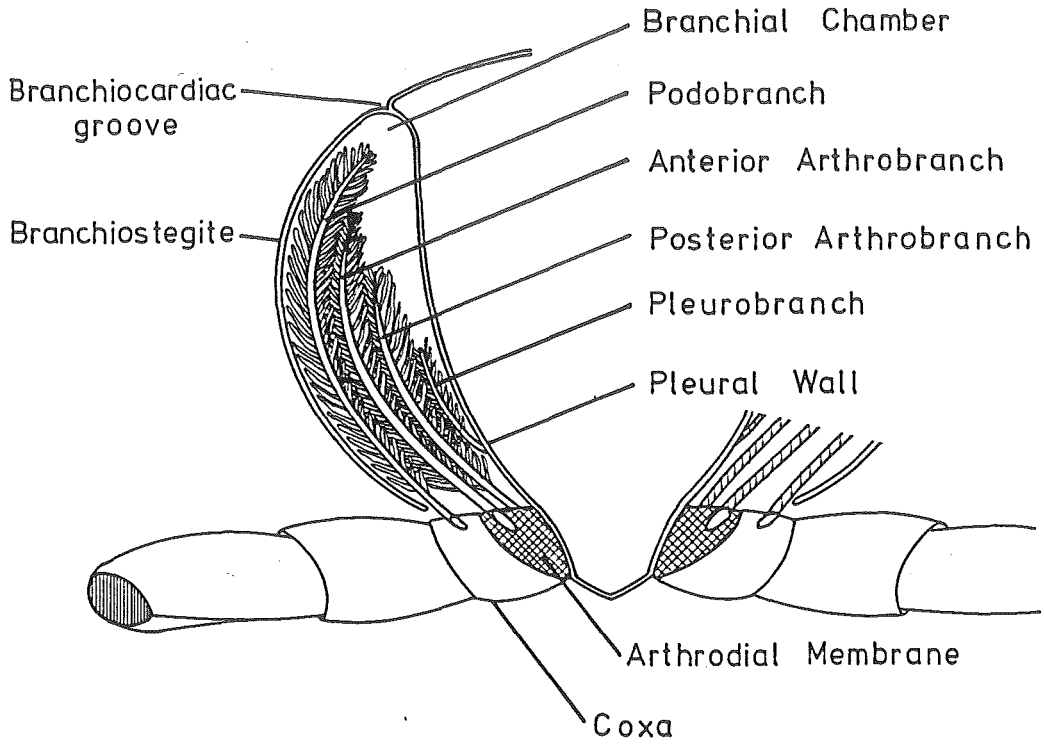


Figure 2.2 Schematic section through the branchial chamber of a parastacoid crayfish to show the disposition of the gills.

Astacus fluviatilis (Huxley, 1880, p78). Laminate epipodites like these are not found in the Parastacoidea.

The classic 1880 text "The Crayfish" by Huxley includes this comment in the description of *Astacus fluviatilis*, "The six podobranchiae, or gills which are attached to the basal joints of the legs ..... differ a good deal in the details of their structure from those which are fixed to the interarticular membranes. At the upper end the stem divides into two parts, that in the front, the plume, resembling the free end of the other gills, and that behind, the lamina, a broad thin plate bent upon itself longitudinally in such a manner that its folded edge lies forwards".

The presence or absence of gills and epipodites and their condition, either rudimentary or developed, provides a gill formula for each species (Table 2.1). The gill formulae for different freshwater crayfish genera demonstrate differences

Table 2.1 Summary of the gill arrangement of *Astacus*, with a gill formula of 18 + 3r + 7ep, (Grove and Newell, 1981). Hobbs, (1974) gives the gill formula for this genus as 18 + 2r + ep or 18 + 3r + ep.

Appendage	Epipodite	Podobranch	Arthrobranch		Pleurobranch
			Anterior	Posterior	
Maxilliped 1	X	O	O	O	O
Maxilliped 2	X	X	X	O	O
Maxilliped 3	X	X	X	X	O
Cheliped	X	X	X	X	O
Walking Leg 1	X	X	X	X	R
Walking Leg 2	X	X	X	X	R
Walking Leg 3	X	X	X	X	R
Walking Leg 4	O	O	O	O	X
Total	7	6	6	5	1 + 3R

X = present; O = absent; R = rudiment

between the superfamilies (Table 1.2). The Parastacoidea are distinguished from the Astacoidea in both the number of gills and the presence of epipodites.

In the Astacoidea the epipod of the podobranchae is plate-like and that of the first maxilliped is devoid of branchial filaments. In the Parastacoidea there is no epipodal plate on the podobranchae and the epipod of the first maxilliped bears a number of well-developed branchial filaments (Hobbs, 1988; Holdich & Reeve, 1988).

In this chapter the morphology of the branchial chamber of *P. zealandicus* is investigated. There are photographs and illustrations to demonstrate the disposition of the gills and their relative size. The fine structure of the gills was studied using light microscopy and electron microscopy. The preliminary results are compared with work on other Parastacoidea, and contrasted with the Astacoidea.

II. MATERIALS AND METHODS

(1) Location and number of gills

The gills and the arthrodial membranes in the branchial chamber of the crayfish are white, which makes structures difficult to see, and a method to colour the tissues was devised. The crayfish were killed by severing the ventral ganglia. About 0.3 ml of haemolymph was removed from the pericardial region and replaced with a red or blue water based food grade colouring agent used in the manufacture of confectionery. The colour was rapidly circulated by the heart which continued beating for some time. This provided a background for the white arthrodial membranes, and assisted in the locating, identifying, viewing, photographing and drawing of the gills and epipodite in the branchial chamber.

The branchiostegite was removed, exposing the gills. The branchial chamber was drawn in three stages; the first view with all the podobranchae visible, the second view was with all the anterior arthrobranchae visible, and the third view with the posterior arthrobranchae, the pleurobranchae and the epipodite visible. The gills were photographed and drawn, with care given to the accurate location, orientation and relative size.

(2) Scanning electron microscopy (S.E.M.)

The crayfish were killed by severing the ventral ganglia. The branchiostegite was removed to uncover the gills, and the podobranchae, arthrobranchae, pleurobranchae and epipodite were cut from the limbs and pleural wall. The tissues were placed in aldehyde fixative (2.5 % glutaraldehyde; 1 % paraformaldehyde) in 0.1 M sodium cacodylate buffer (pH 7.3) for 3 days at 4°C. This was followed by an overnight rinse in sodium cacodylate buffer. The specimens were then post-fixed in 2 % buffered osmium tetroxide for 1 day at 4°C. After another overnight rinse in the buffer solution, the specimens were dehydrated in a graded alcohol series; 50 - 100 % ethanol, for about 2 hours in each solution and a final period overnight in 100 % ethanol.

The gills were then transferred to amyl acetate through

a four-step ethanol/amyl acetate series (2 hours in each solution, and overnight in a fresh solution of 100 % amyl acetate). After drying in a liquid CO_2 critical point drier, the specimens were mounted on aluminium stubs using conductive carbon paint. Finally they were sputter coated with 60 nm of gold and observed with a Jeol JSM-6100 scanning electron microscope at accelerating voltage of 3 - 15 kV.

(3) Transmission electron microscopy (T.E.M.)

The crayfish were killed and dissected as for the SEM, and the tissues were immediately placed in the primary fixative (2.5 % glutaraldehyde, 1 % paraformaldehyde, 0.2 M sucrose, in 0.1 M sodium cacodylate buffer adjusted to pH 7.3). Each gill was cut into small ≈ 1-2 mm long portions. After fixation for 2 - 4 hours at 4°C, excess fixative was removed with three 10 minute washes in the buffer at 4°C. The tissues were then post fixed in 1 % buffered osmium tetroxide for 1 - 3 hours (at 4°C), followed by three further 10 minute washes in buffer. Dehydration was achieved using a graded alcohol series (30, 50, 70, 80, 90, 95, 100%), 15 to 20 minutes in each solution, and two times in absolute ethanol.

The alcohol was replaced with acetone (the transitional solvent) by washing twice in absolute acetone for 20 minutes. The tissues were then infiltrated with Spurr's epoxy resin, using the following resin/acetone series; 50 % resin - 2 to 3 hours, 75 % resin - 3 to 4 hours, 90 % resin - overnight, and pure resin for 1 to 2 hours. The specimens were then placed in fresh 100 % resin in plastic moulds and cured at 65°C for 12 to 36 hours. The blocks were left for at least one day before cutting. Semi-thick and ultrathin sections were cut on an LKB Bromma 8800 ultramicrotome. The semi-thick sections were 2 µm, and were placed on glass slides and stained with 0.5 % toluidine blue (in borax). Gold to silver thin sections (50 to 100 nm) were cut and placed on 200 mesh copper grids. These were stained with 2 % uranyl acetate (in 50% ethanol) for 30 minutes, followed by lead citrate for 15 minutes, and then observed with a Jeol JEM 1200-EX TEM at an accelerating voltage of 80 kV.

Problems obtaining well-embedded tissues were encountered, so longer times than normal for fixation and resin infiltration were tried.

(4) Light microscopy

The semi-thin sections, prepared and cut as for the T.E.M. above, were made into permanent mounts using D.P.X. mountant fluid and thin glass coverslips, and were then photographed using an Olympus BH2 light microscope.

(5) Measuring the cuticle thickness

Micrographs taken with the T.E.M. were used to measure the thickness of the cuticle. The cuticle thickness from gills which differed morphologically was compared with a Student's t test.

III. RESULTS

(1) The location and number of gills

The gill formula for *P. zealandicus* is: 20 gills, one rudimentary gill, and one epipodite. The summary of the gill arrangement can be seen in Table 2.2.

The podobranchae of *P. zealandicus* are of sufficient length to reach the dorsal carapace within the branchial chamber, Figure 2.3. The progressive removal of the gills indicated that the whole of the branchial space was utilised, Figure 2.4. The gills and epipodite are identified in Figures 2.5, 2.6 and 2.7.

(2) The external morphology

The podobranchae have a "narrow membranous wing" (Hopkins, 1970) extending from the base of the stem along half the length of the stem (Figures 2.3 and 2.8). There are setae towards the outer edge of the membrane which are covered with small serrated scales for the distal half of their length (Figures 2.8, 2.9 and 2.10).

The gill stem is near vertical (Figures 2.3 to 2.7), and the filaments which are perpendicular to the stem proximally,

curve upwards to lie nearly parallel to the stem. In cross section the stem of the podobranchae appears to resemble a "D" with filaments over the whole of the curved surface (Figure 2.8), and a few shorter filaments on the broad flat surface (Figure 2.18). The flat surface of the gill faced the posterior at an angle of approximately 45° to the mid-line. This can be seen in Figure 2.3 in the attitude of the membrane attached to the stem.

The stems of the anterior arthrobranchae, posterior arthrobranchae and pleurobranchae, with the exception of the posterior arthrobranch on the third walking leg (segment 7), are all oval or crescent in transverse section. One face of the stem is covered in filaments and the other face (which is the concave face of the crescent), has only a few filaments. The posterior arthrobranch on segment 7 has only two filaments. The epipodite on the first maxilliped has gill filaments on one side (Figure 2.11).

Three filament types have been identified. There are filaments which have rounded ends without a spine or hook (Figures 2.12 and 2.13). The second type have conical ends which terminate in a curved spine (Figures 2.14 and 2.15) or a hook (Figure 2.16). There are some filaments on the

Table 2.2 Summary of the gill arrangement of *Paranephrops zealandicus*, with a gill formula of 20 + 1 rudimentary + 1 epipodite.

Appendage	Podobranch	Arthrobranch		Pleurobranch
		Anterior	Posterior	
Maxilliped 1	E	O	O	O
Maxilliped 2	X	X	O	O
Maxilliped 3	X	X	X	O
Cheliped	X	X	X	O
Walking Leg 1	X	X	X	X
Walking Leg 2	X	X	X	X
Walking Leg 3	X	X	R	X
Walking Leg 4	O	O	O	X
Total	6 + E	6	4 + R	4

X = present; O = absent; R = rudiment; E = epipodite.

Figure 2.3 A photograph of the right side of *P. zealandicus* with the branchiostegite removed and the podobranchae exposed. The crayfish has been injected with a red food colouring to improve visualisation, and the preparation is floating in water to suspend the gills. The exposed area is 5 cm long. Each podobranch has a narrow membranous lamina at the base, and these assist in locating the individual podobranchae. The podobranchae are, from the left, on walking leg 3, walking leg 2 and walking leg 1, the cheliped, maxilliped 3 and then maxilliped 2. The parts are all labelled in the drawings on the following pages.

epipodite which have two curved spines (Figure 2.17). Some filaments have been found which have conical ends which terminate in a protuberance (Figure 2.19) which may be another form of spine, or a transition stage between the two filament types already mentioned. The distribution of the filament types is unclear.

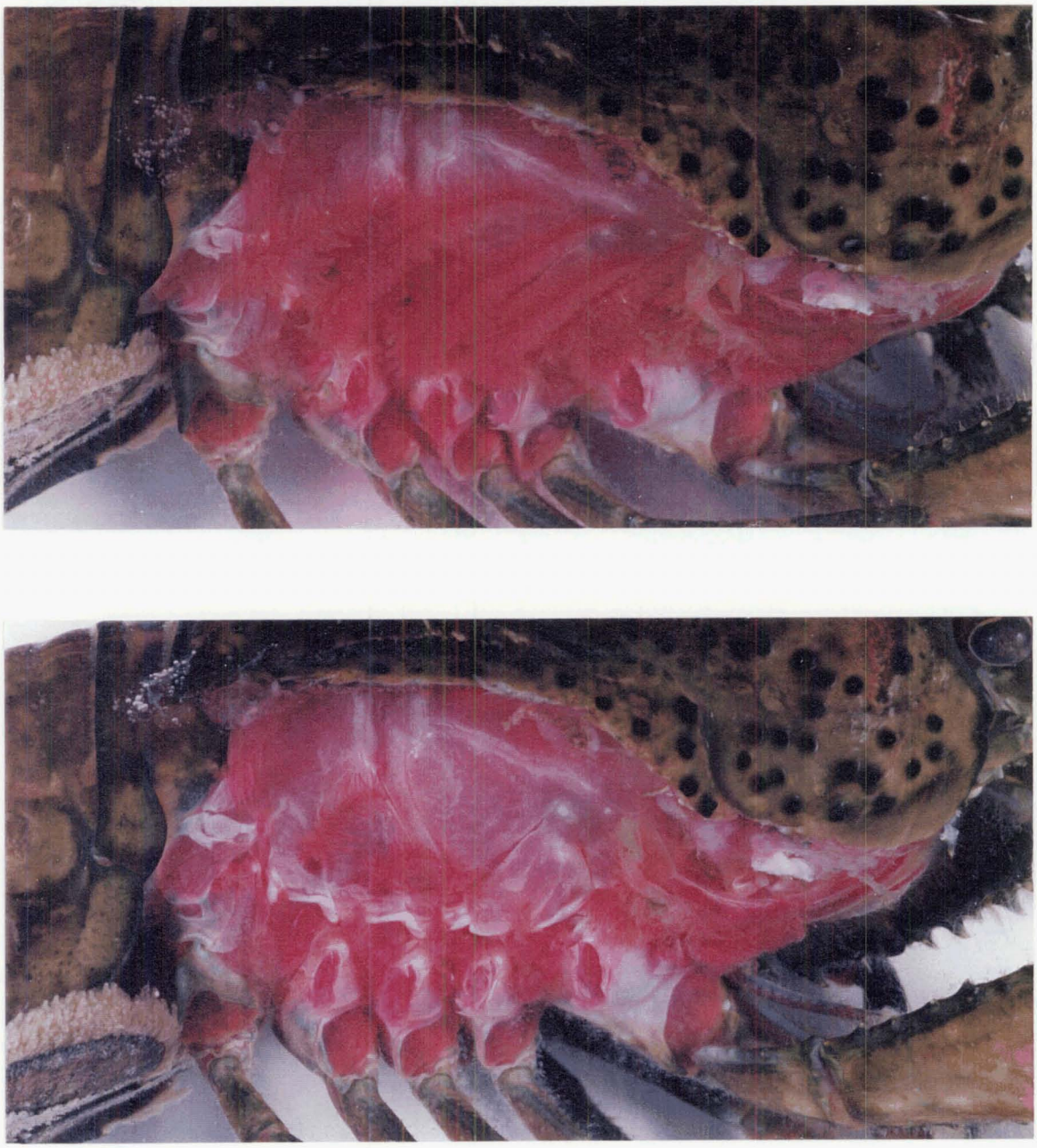


Figure 2.4 Top The right side of *P. zealandicus* with the branchiostegite and podobranchae removed and the anterior arthrobranchae exposed. The pleurobranch of walking leg 4 is visible on the extreme left above the fourth walking leg. **Bottom** The right side of *P. zealandicus* with the branchiostegite, podobranchae, anterior arthrobranchae and pleurobranch of walking leg 4 removed. The posterior arthrobranchae and the remaining pleurobranchae are exposed. The epipodite is visible on the right, below the scaphognathite. The parts are all labelled in the drawings on the following pages. The exposed area is 5 cm long, and the preparation was the same as for Figure 2.3.

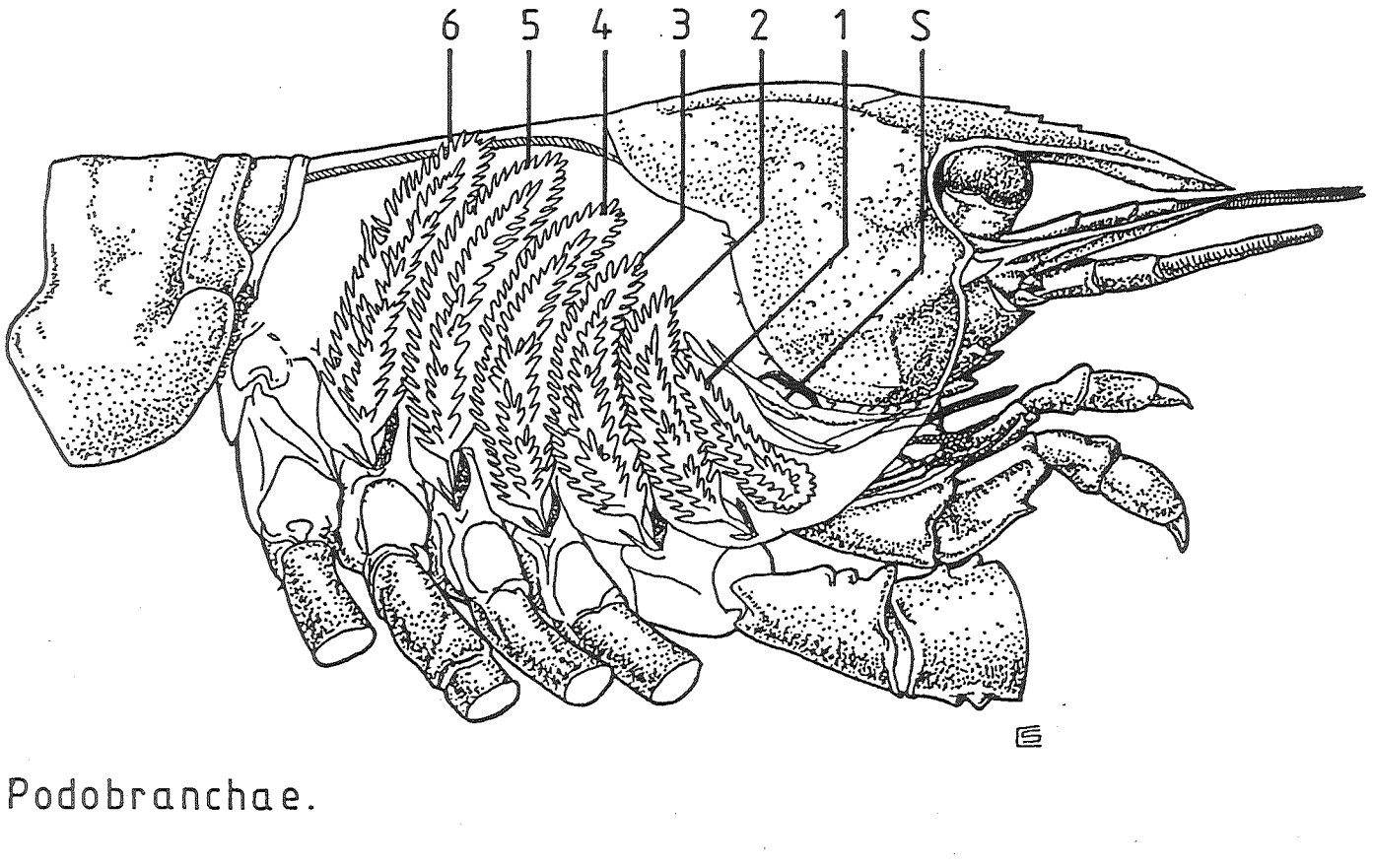


Figure 2.5 A drawing of the right branchial chamber of the crayfish *Paranephrops zealandicus* after the branchiostegite has been removed. The labelled parts are the podobranchae of the following limbs : **1** = maxilliped 2, **2** = maxilliped 3, **3** = cheliped, **4** = walking leg 1, **5** = walking leg 2, **6** = walking leg 3, and **S** = scaphognathite.

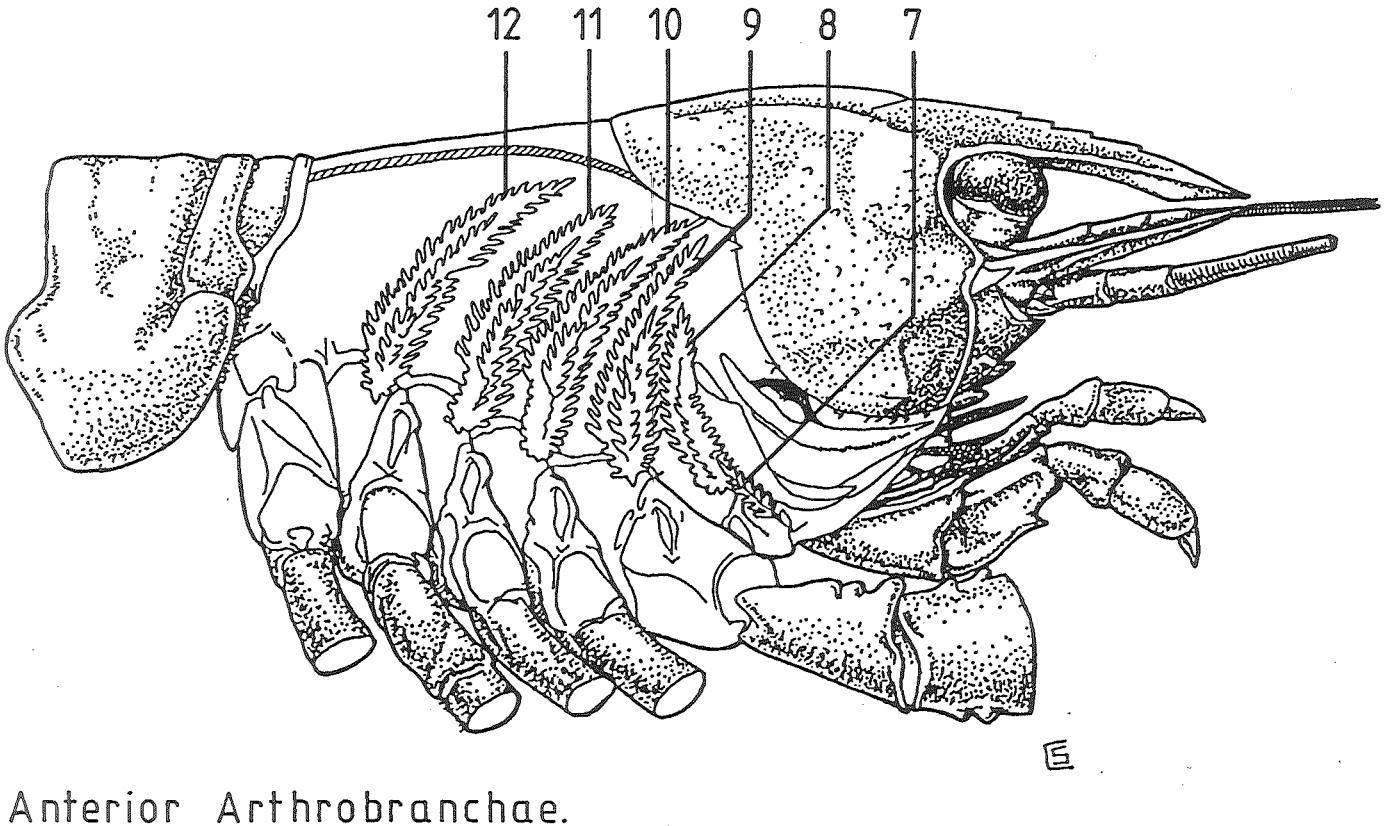


Figure 2.6 A drawing of the right branchial chamber of the crayfish *Paranephrops zealandicus* after the branchiostegite and the podobranchae have been removed. The labelled parts are the anterior arthrobranchae of the following limbs : **7** = maxilliped 2, **8** = maxilliped 3, **9** = cheliped, **10** = walking leg 1, **11** = walking leg 2, and **12** = walking leg 3.

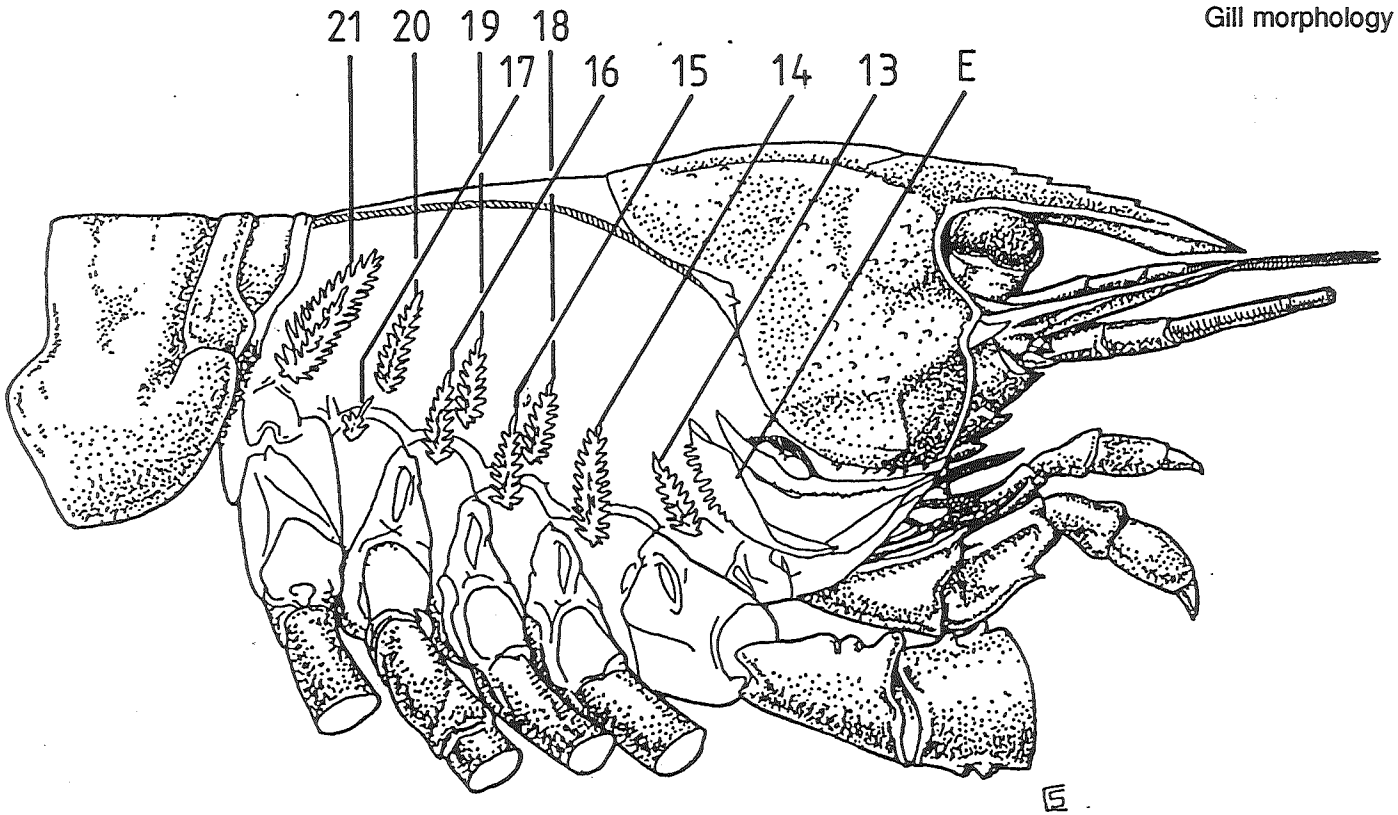


Figure 2.7 A drawing of the right branchial chamber of the crayfish *Paranephrops zealandicus* after the branchiostegite, podobranchae and anterior arthrobranchae have been removed. The labelled parts are the posterior arthrobranchae of the following limbs : **13** = maxilliped 3, **14** = cheliped, **15** = walking leg 1, **16** = walking leg 2, **17** = rudimentary gill on walking leg 3. The pleurobranchae are : **18** = walking leg 1, **19** = walking leg 2, **20** = walking leg 3, **21** = walking leg 4, and **E** = the epipodite.

Figure 2.8 A scanning electron micrograph of a podobranch from the first walking leg (5th thoracic segment) in the left branchial chamber. Some of the filaments at the end of the stem (top of figure) and the outer margin of the stem (right of figure) bear hooked spines, the remainder of the filaments have round tips. The podobranch has a narrow membranous lamella which extends from the base of the stem, (bottom left), for approximately half its length. The lamella has both fine and very coarse setae.

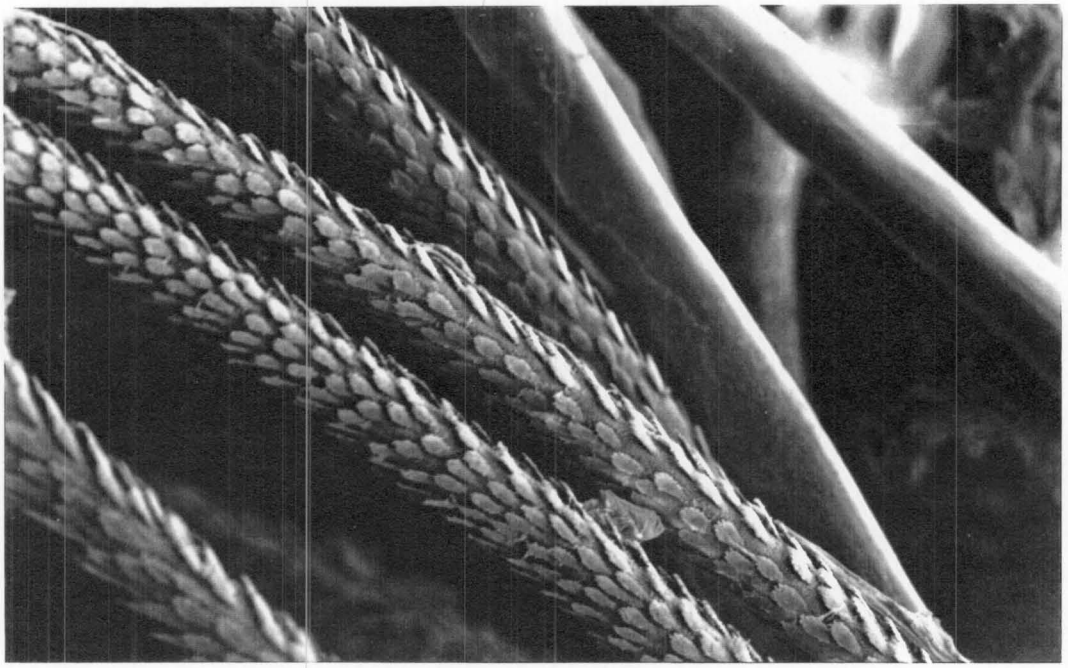


Figure 2.9 A high power view of the fine setae on the edge of the lamella on a podobranch stem (see Figure 2.8). Small serrated scales cover the distal half of the setae. In the right background are segments of setae without scales.

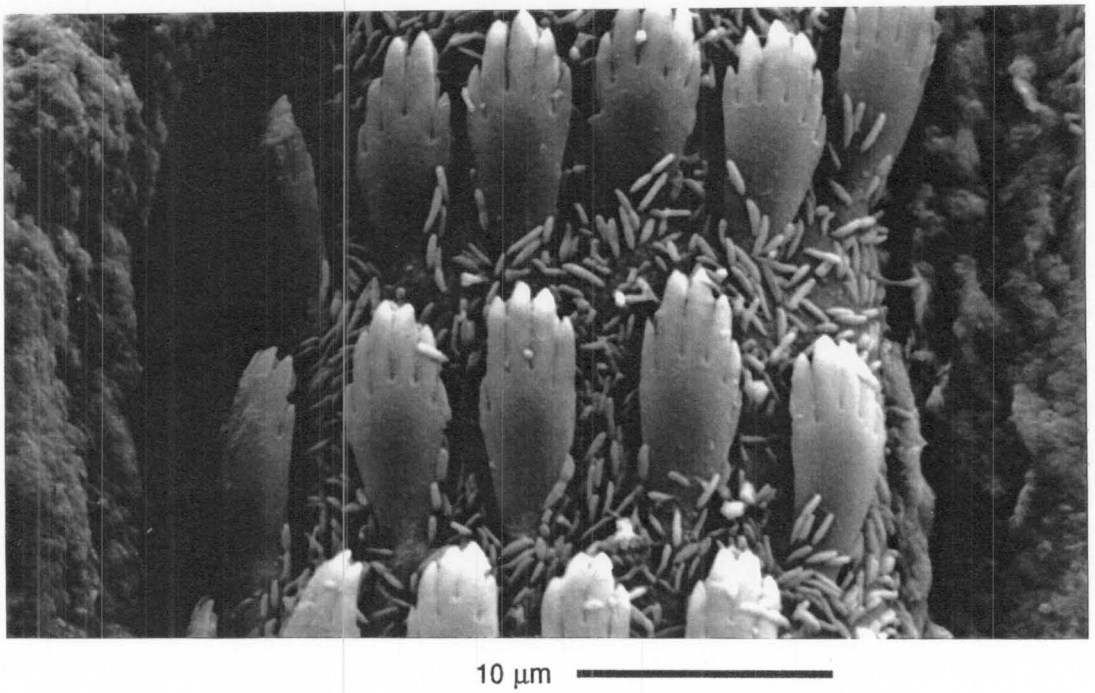
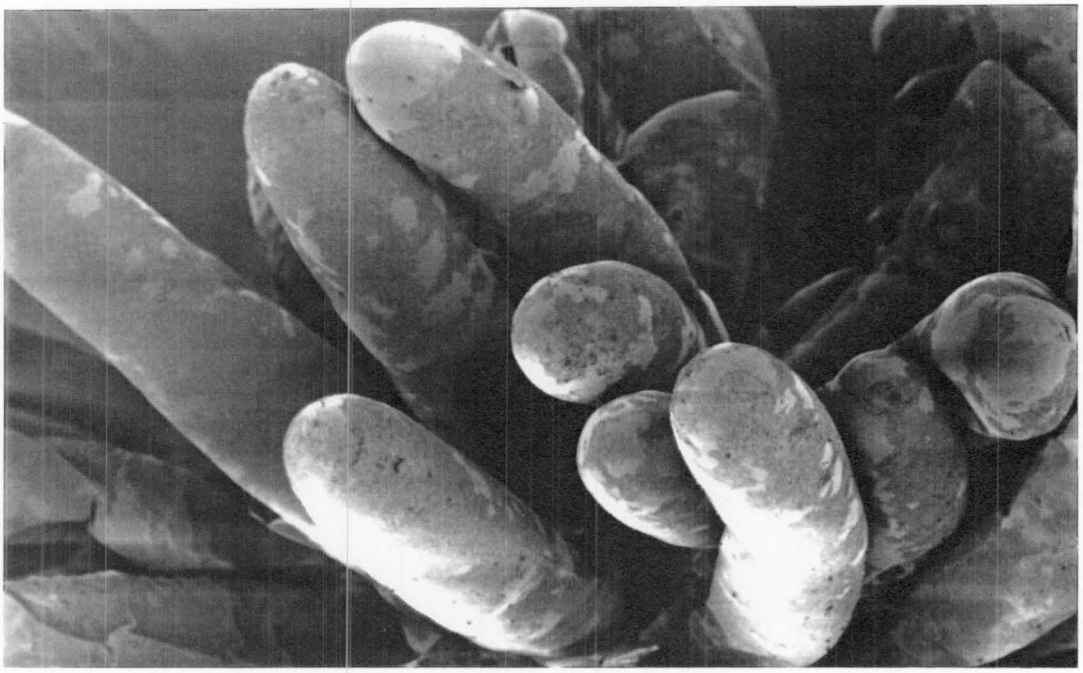


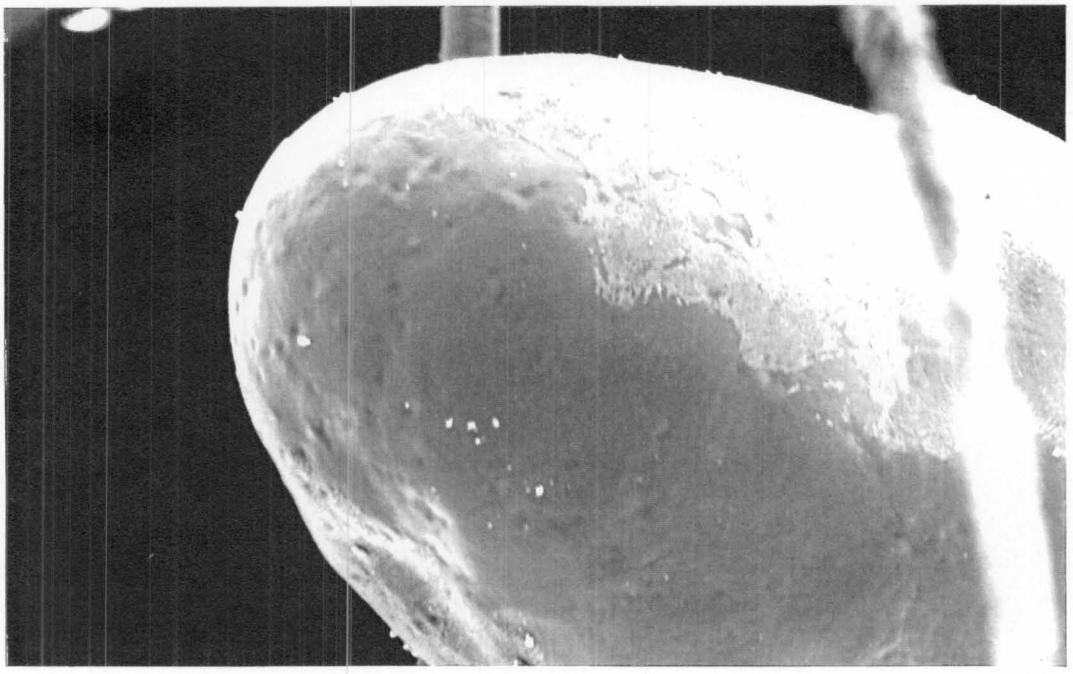
Figure 2.10 The serrated scales on the setae highly magnified. There is a dense population of bacteria covering the stem.

Figure 2.11 A micrograph of the epipodite from the right branchial chamber. It is attached to the first maxilliped (1st thoracic segment). Filaments cover 1/3 of the surface in well defined strips. All the filaments terminate in a curved or hooked spine.



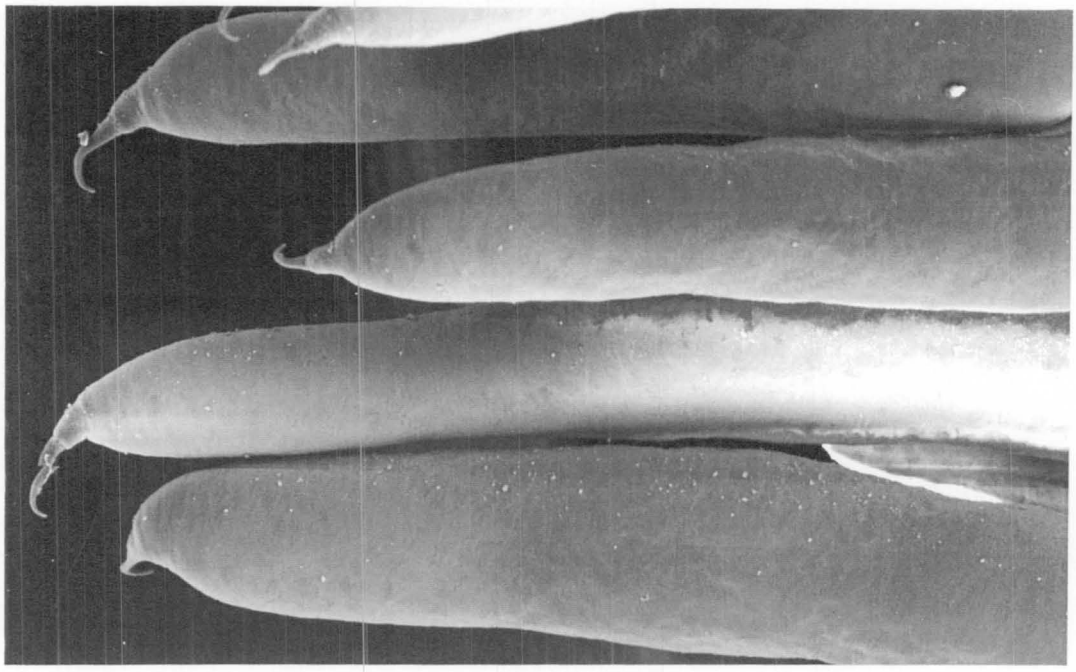
100 μm

Figure 2.12 Gill filaments with rounded ends and no hooks or spines, from the middle of the shaft of an anterior arthrobranch. The filament surface appears smooth.



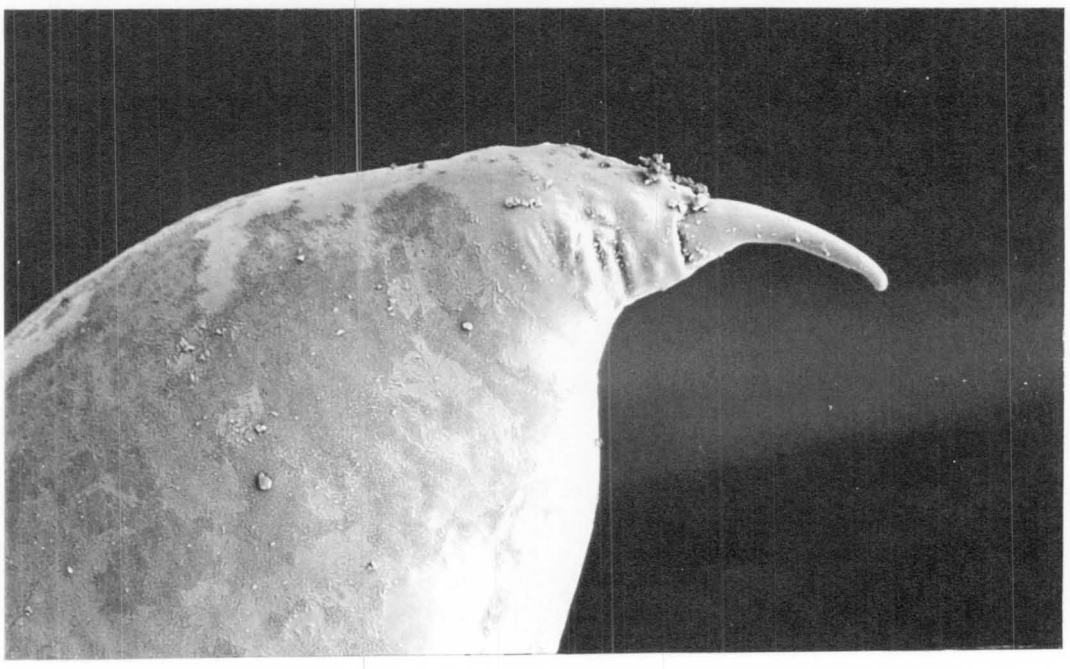
10 μm

Figure 2.13 High magnification of the tip of a rounded gill filament.



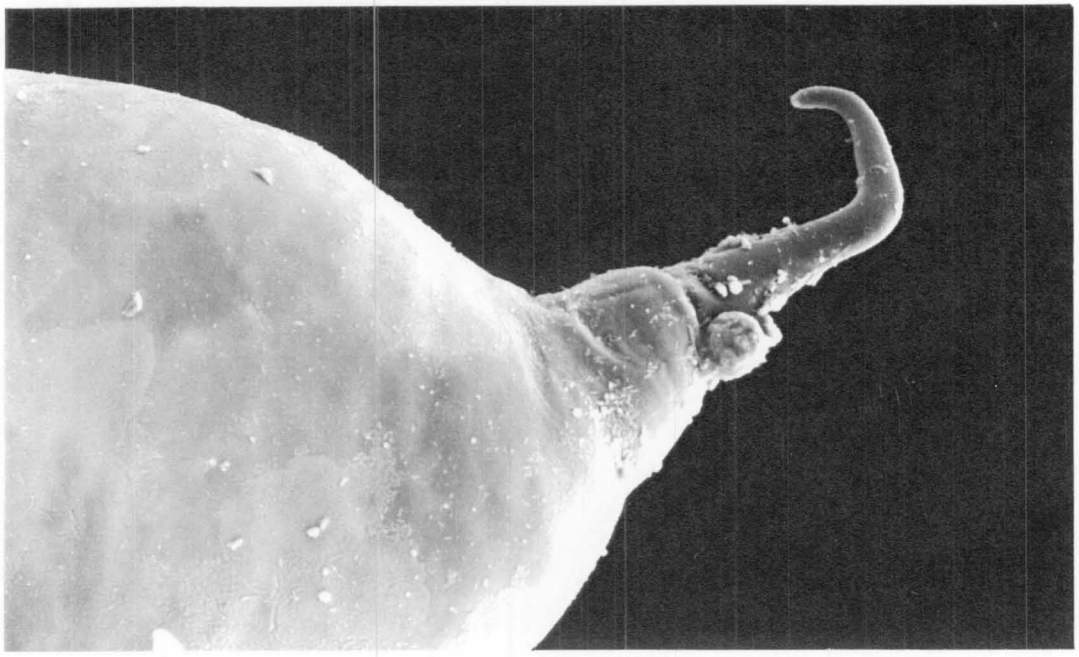
100 µm

Figure 2.14 A group of gill filaments terminating in spines. These filaments have a conical rather than a rounded end and are found on podobranchae and epipodites.



10 µm

Figure 2.15 A high magnification of the curved spine on the end of a gill filament.

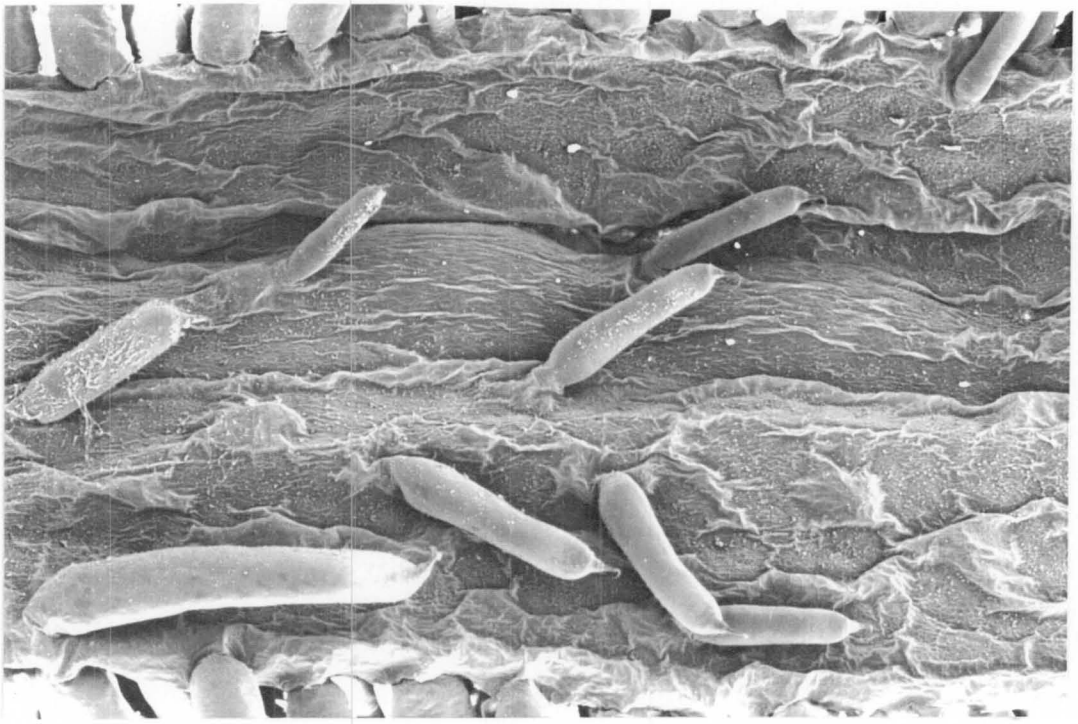


10 µm

Figure 2.16 A high magnification of a hook on the end of a gill filament.

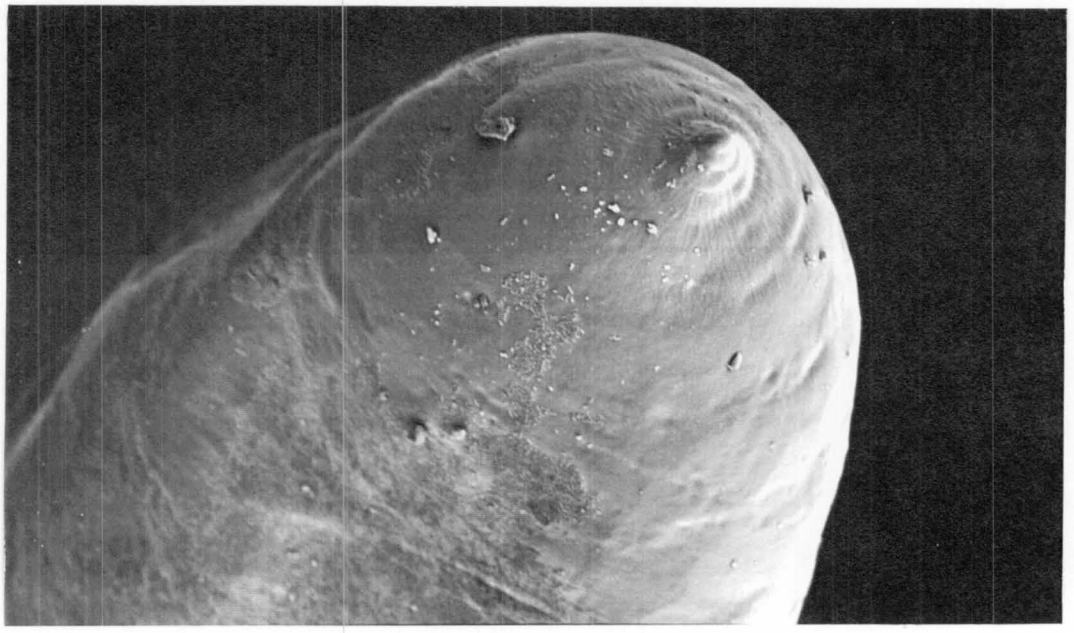
10 µm

Figure 2.17 One of the many small filaments found on the surface of the epipodite. All the filaments on the epipodite have a spine, and a few have two like this specimen.



100 µm

Figure 2.18 The flat side of a podobranch stem faces towards the posterior. It has only a few gill filaments which are short and appear to terminate in a hook.



10 µm

Figure 2.19 Some of the gill filaments were neither round and smooth, nor tapered and hooked, but possessed a small protuberance.

(3) The fine structure

The light microscope micrographs of the thick transverse sections, Figures 2.20, 2.21 and 2.22, indicated that there were two different structural types of gill filaments. The preliminary sections did not indicate a change in morphology along the length of the filaments. In one type of filament the external cuticle was lined with a layer of epithelial tissue, with a total thickness of 7 - 10 μm, and with large cell bodies up to 35 μm deep. The central region of this filament contained an afferent and an efferent branchial vessel, separated by a septum (Figure 2.20), with haemolymph lacunae between the tissue lining the cuticle, the large cell bodies and the afferent and efferent vessels.

The other filament type also had an afferent and an efferent vessel. The epithelial layer was much thinner, and the total thickness, including cuticle, was often no more than 1.5 to 2 μm (Figure 2.21). The cell bodies were considerably smaller and consequently the lacunae were larger and more extensive. The three transverse sections, Figures 2.20, 2.21 and 2.22 were approximately 200 μm in diameter.

The cuticle was continuous around the circumference of the filament (Figure 2.22). The large cell bodies were epithelial flange cells or pear cells (Taylor and Taylor, 1992). The flange cells had a distinct pear shape, a very prominent nucleus (Figure 2.22), many mitochondria, large quantities of rough endoplasmic reticulum, and the apical membrane in contact with the cuticle was folded to produce many apical leaflets (Figure 2.23). Spongy connective tissue surrounded the basal part of the flange cell.

There was a highly significant difference between the cuticle thickness of the two filament types. The filament with a thick epithelial layer against the cuticle and large flange cells had a cuticle thickness of 1220 ± 177 nm (± 1 sd, n = 22). The filament with the thin epithelial layer against the cuticle had a cuticle thickness of 714 ± 171 nm (n = 20). A two tailed Student's t test confirmed that the two cuticle types were significantly different ($P < 0.001$, $t_{calc.} = -9.41$, 39 df). The thickness of the two different cuticle types can be compared in Figures 2.24 and 2.25. Both

micrographs have the same scale, which can be confirmed by the bacteria on the external surface of both the filaments. The cuticle thickness can also be compared in Figures 2.26 and 2.27.

The thin cuticle had a very thin layer of epithelial tissue attached (Figures 2.21 and 2.24). This approximately doubled the total distance between the outside of the filament and the haemolymph within the lacunae. The tissue attached to the thicker cuticle was much thicker (Figures 2.20 and 2.27).

There was considerable difference in the flange cells associated with thick cuticle and with thin cuticle. In the filament with thin cuticle, the tissue near the neck region of a flange cell had few mitochondria and the apical leaflets were not extensive (Figure 2.26). In contrast to this, in a filament with thick cuticle the tissue at the neck and flange regions of a flange cell was packed with mitochondria, and the apical leaflets were well developed (Figure 2.27). They were considerably elongated and penetrated deep into the cell cytoplasm (Figure 2.28). At the base of the apical leaflets there were many small vesicles. There were also extensive basolateral infoldings which enveloped the mitochondria and often extended from the basal membrane almost to the apical leaflets. The deep basolateral infoldings increase the membrane surface area of the cell considerably (Figure 2.29). Adjacent flange cells are joined by septate desmosomes which provide a large area of adhesion between cells (Figure 2.27). Extensive rough endoplasmic reticulum, numerous Golgi bodies and large quantities of glycogen were also associated with the epithelial flange cells of filaments with a thick cuticle (Figures 2.30 and 2.31).

IV DISCUSSION

The gill formula for the genus *Paranephrops* was reported by Hopkins (1970) and was confirmed for *Paranephrops zealandicus* as: 20 gills, one rudimentary gill, and one epipodite. *P. zealandicus* does not have epipodites on the

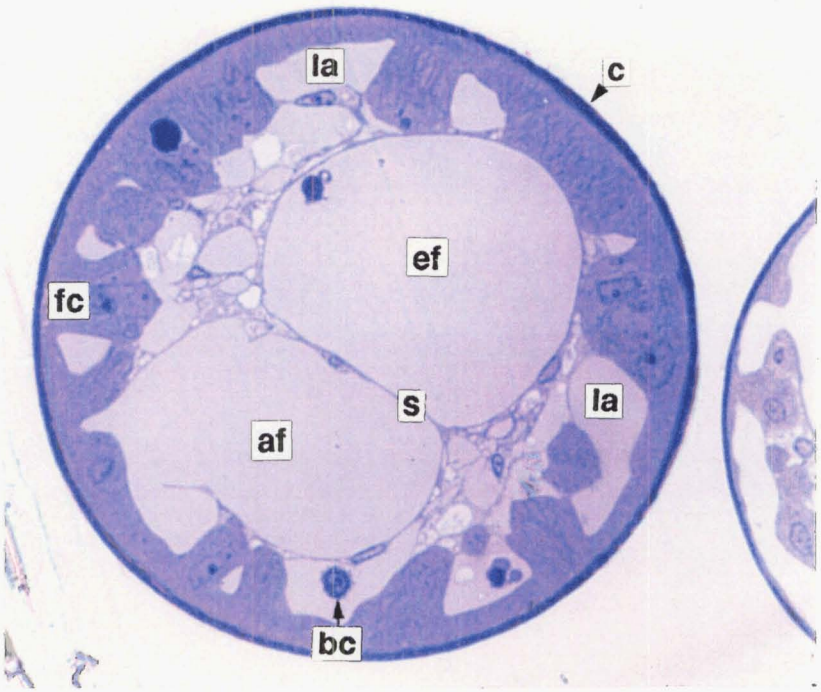


Figure 2.20 A transverse section of an ion regulating filament showing the afferent branchial vessel (**af**), efferent branchial vessel (**ef**), lateral haemolymph lacunae (**la**), epithelial flange (pear) cells (**fc**), cuticle (**c**), blood cell (**bc**), and the septum (**s**) which separates the afferent and efferent vessels. (Note the gill section partially visible on the right of the figure is presented below).

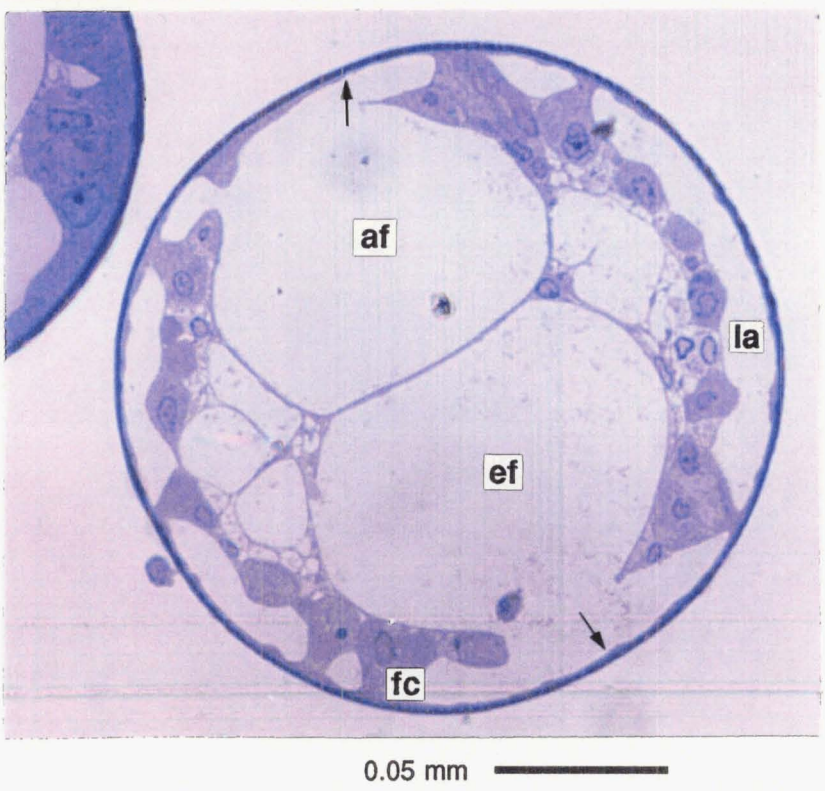


Figure 2.21 A transverse section of a respiratory filament with the afferent vessel (**af**), efferent vessel (**ef**), lacunae (**la**), and epithelial flange cells (**fc**) evident. The lateral cytoplasmic extensions of the flange cells are extremely thin in this section (arrows), minimising the water to haemolymph diffusion distance.

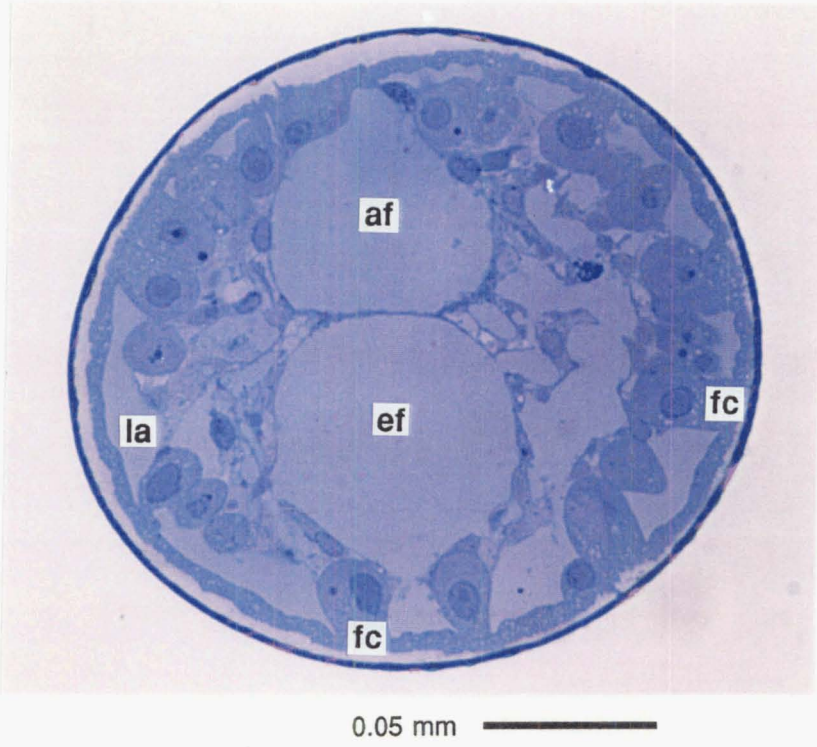


Figure 2.22 A transverse section of an ion regulating filament showing clearly the pear-shaped flange cells (**fc**), some with prominent nuclei. Afferent vessel (**af**), efferent vessel (**ef**), lacuna (**la**). (Note: the epidermal cells have detached slightly from the cuticle during processing).

stems of the podobranchae as described for the Astacoidea, but only has the one epipodite on the first maxilliped. This difference is one of the characters used to separate the Astacoidea from the Parastacoidea. Another difference is the usual presence of gill filaments on the epipodite of the Astacoidea and their absence from the epipodite of the Parastacoidea.

The podobranchae have a narrow membranous wing (Hopkins, 1970), which, starting from the base of the stem, is attached to half the length of the stem. Little is made of this in the general literature, although Swain *et al* (1988) mention the flattened bases of the podobranchae of *Parastacoides tasmanicus* and *Astacopsis franklinii*. They indicate that the flattened bases have setae towards their outer edge, and that the setae are covered with small serrated scales for the distal half of their length. Similar setae have also been found in *P. zealandicus*.

There is still considerable speculation about the functions of the epipodites in the Astacoidea. In the case of *P. zealandicus*, the possibility that the membrane attached

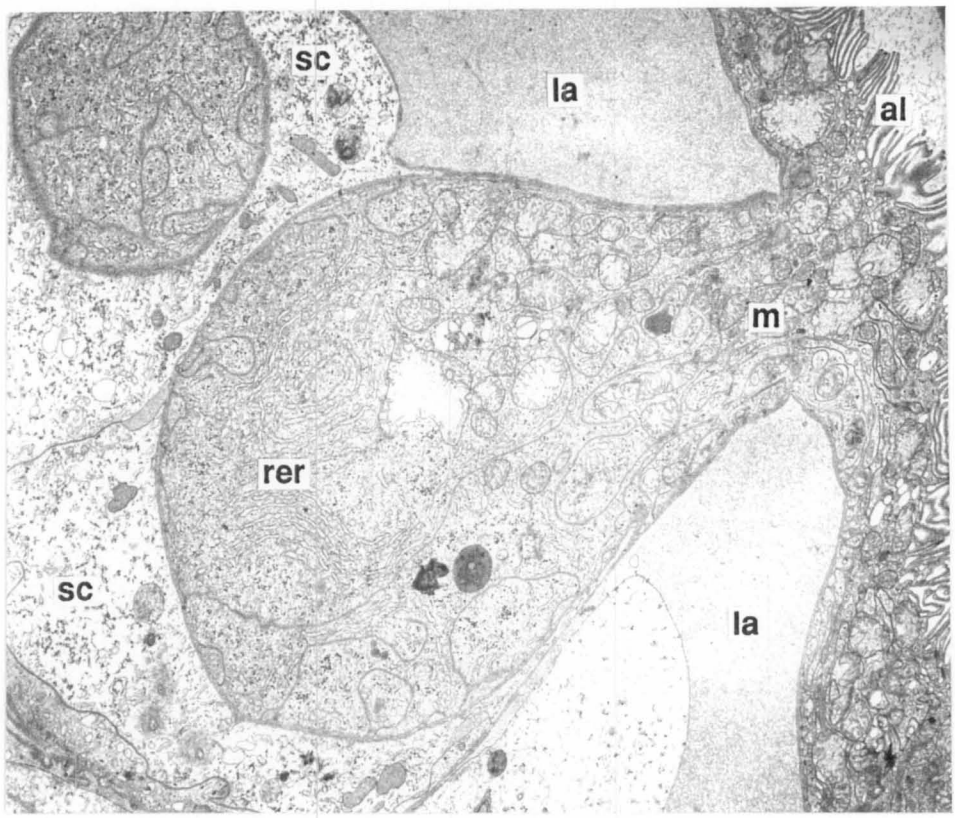
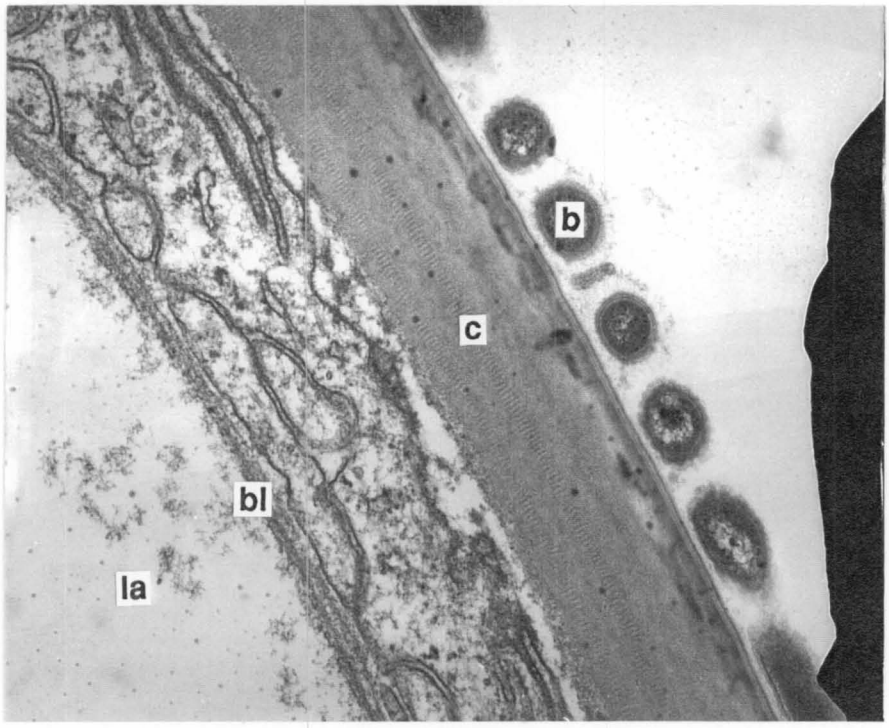


Figure 2.23 A transmission electron micrograph of a flange cell with expanded perikaryon and narrow neck region, with clearly visible mitochondria (**m**) and rough endoplasmic reticulum (**rer**). Note also the apical leaflets (**al**), lacunae (**la**), and spongy connective tissue (**sc**).

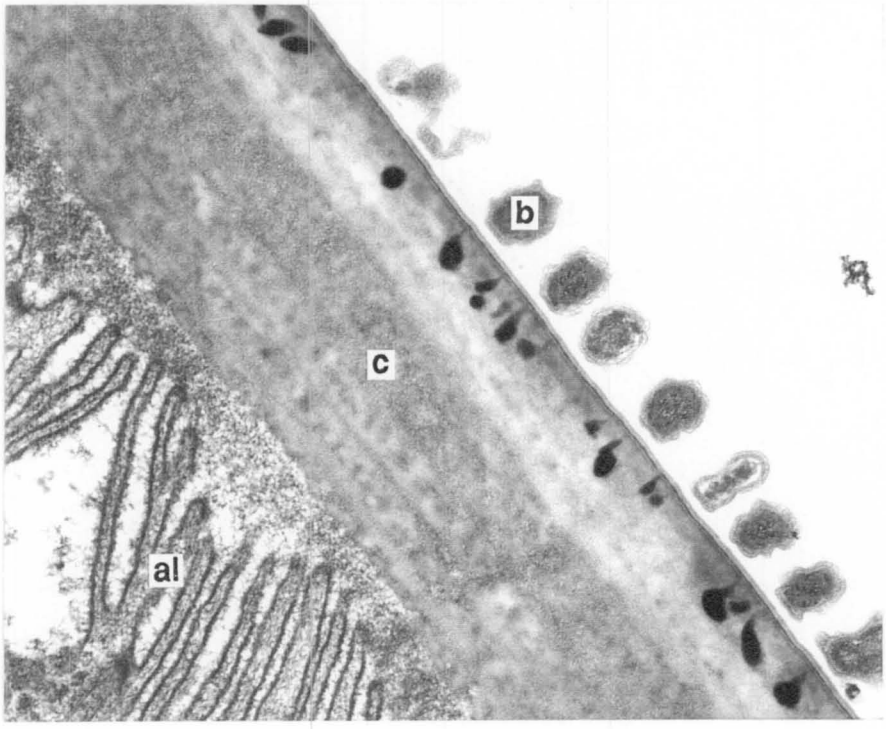
to the stem of the podobranch provides structural support around the bend of the stem cannot be discounted. This would be of considerable advantage to the crayfish when it is out of the water and has lost the support provided by the more buoyant aquatic medium. No investigations appear to have been undertaken on the probable involvement of these membranes in gas exchange or ionic regulation in the Parastacoidea.

The gill filaments have smooth rounded ends, conical ends which terminate in a hook or a curved spine, conical ends which terminate in a protuberance and a few epipodite filaments have two curved spines. The distribution of the filament types is unclear. Swain *et al* (1988) mention that the gill filaments of *A. franklinii* and *P. tasmanicus* often terminate in spines. The number, shape, length and location of the spines is different for both species, and it is



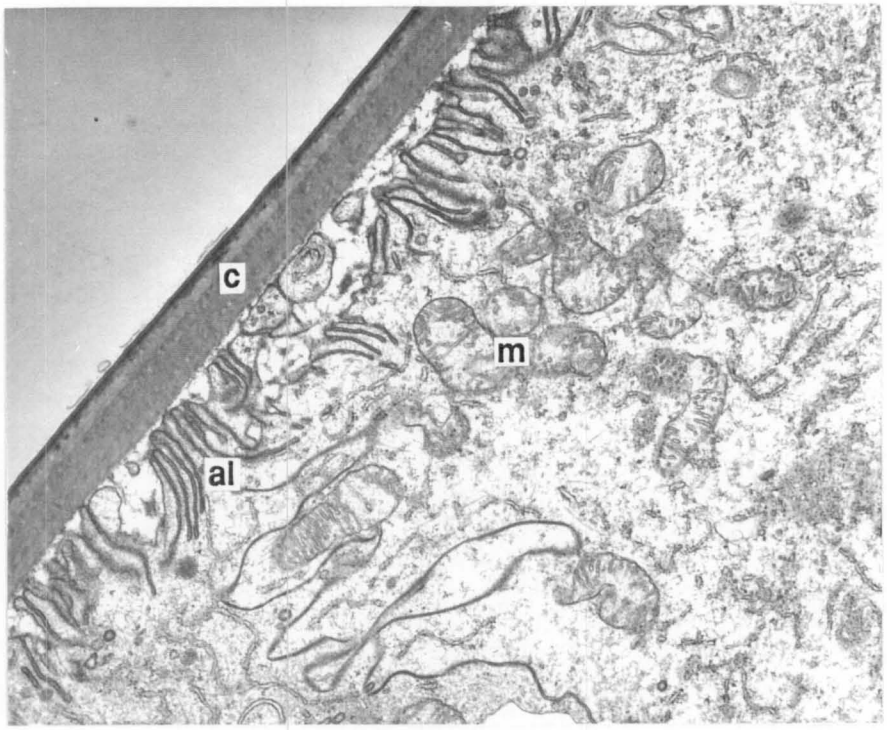
200 nm

Figure 2.24 The cuticle (**c**) of a respiratory filament and the very thin layer of tissue separating it from the haemolymph lacuna (**la**). Note the basal lamina (**bl**) and the bacteria (**b**) attached to the outside of the filament.



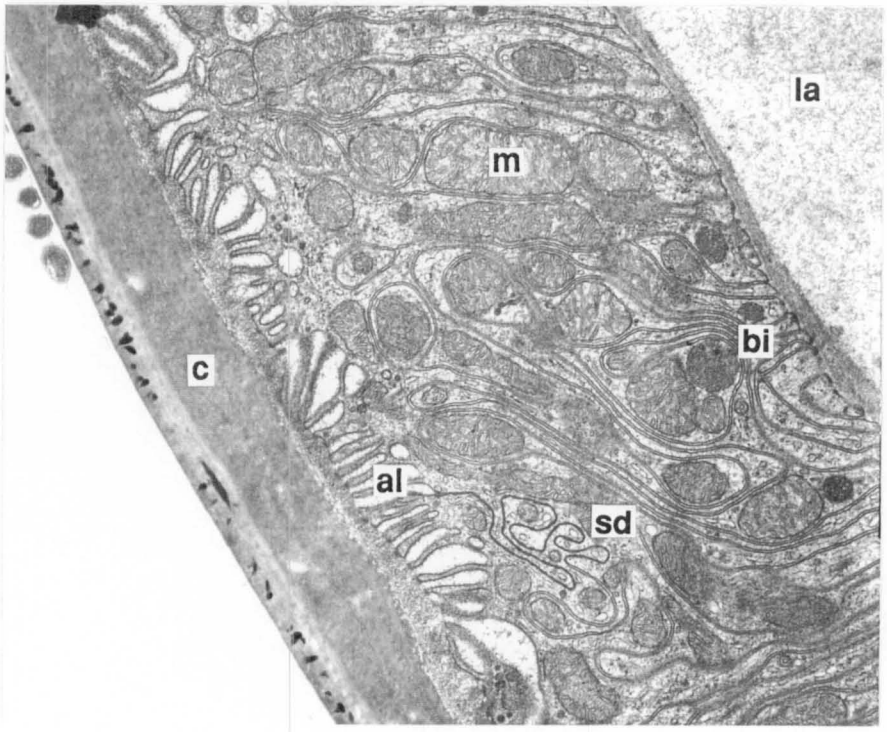
200 nm

Figure 2.25 The cuticle (**c**) of an ion regulating filament which is almost twice the thickness of the cuticle in the above micrograph of a respiratory filament. Note both micrographs are the same scale (refer also to the size of the bacteria **b**). Apical leaflets (**al**) are apparent.



500 nm

Figure 2.26 The thin cuticle (**c**) and underlying tissue of a respiratory filament at the neck region of an epithelial flange cell. The apical leaflets (**al**) are more extensive than in the flange regions (cf. Figure 2.24) and there are more mitochondria (**m**).



500 nm

Figure 2.27 The thicker cuticle (**c**) of an ion regulating filament in the flange region of an epithelial cell. There are high densities of mitochondria (**m**), basolateral infoldings (**bi**), and apical leaflets (**al**), and a septate desmosome (**sd**) between adjoining cells. Haemolymph lacuna (**la**).

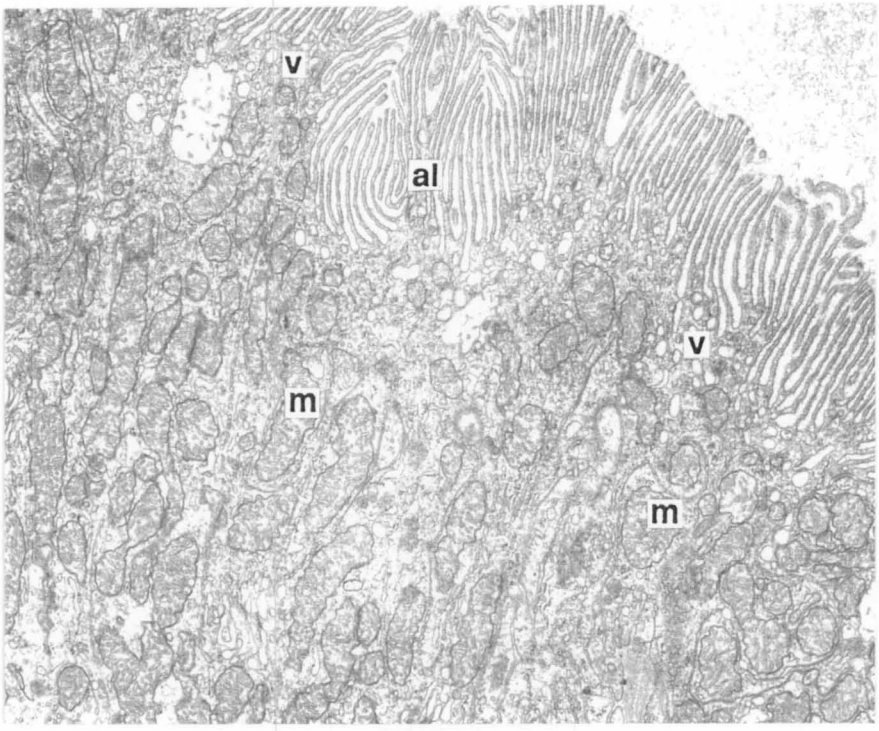


Figure 2.28 The neck region of a flange cell in an ion regulating filament showing elongated apical leaflets (**al**) and numerous mitochondria (**m**). Small vesicles are present at the base of the leaflets (**v**). (The apical region of the cell has detached from the cuticle)

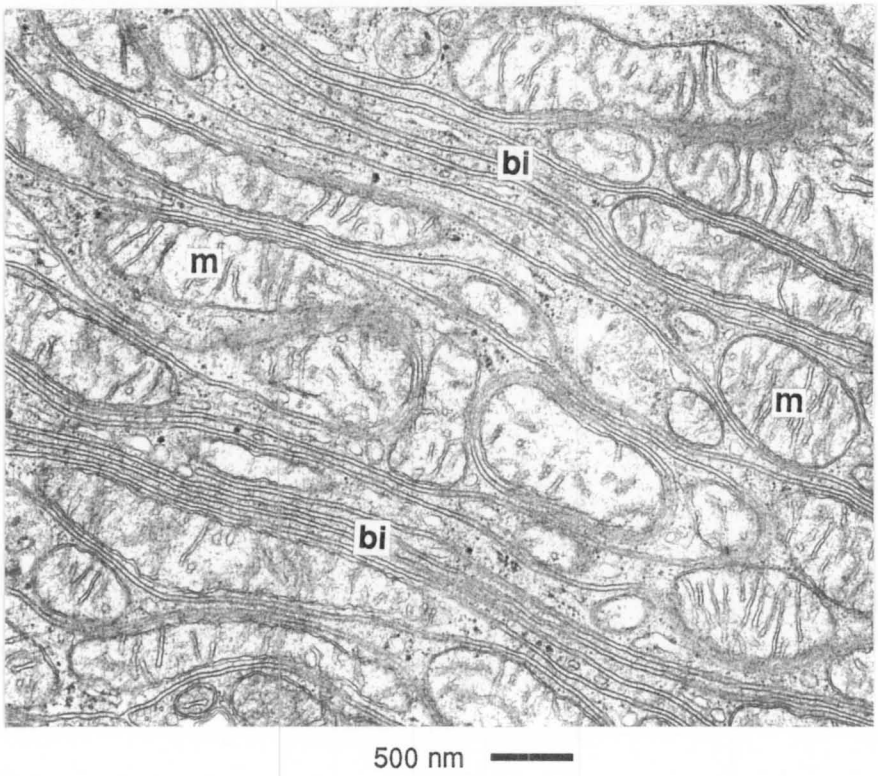
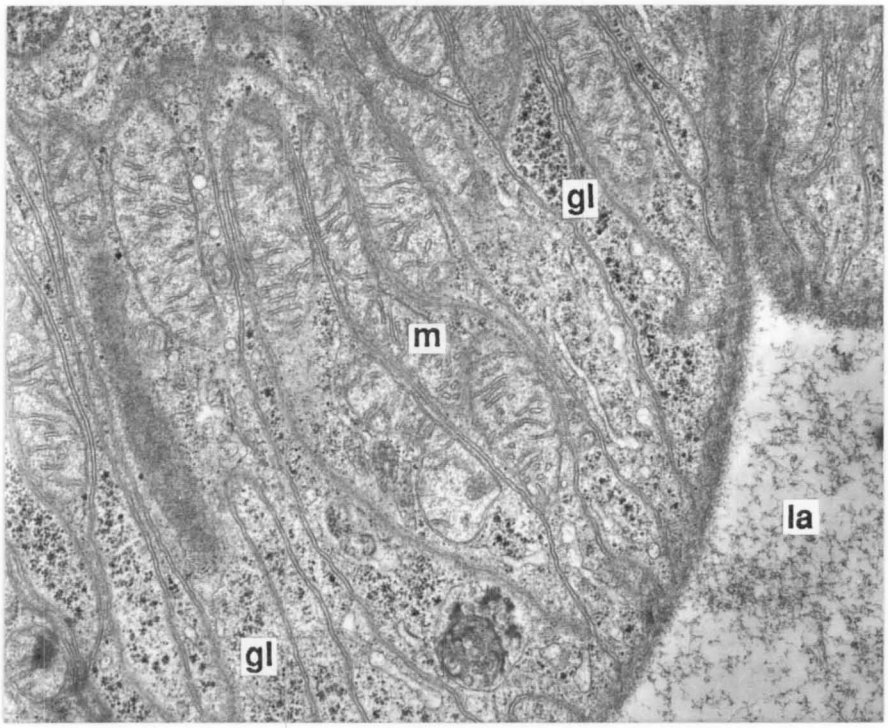
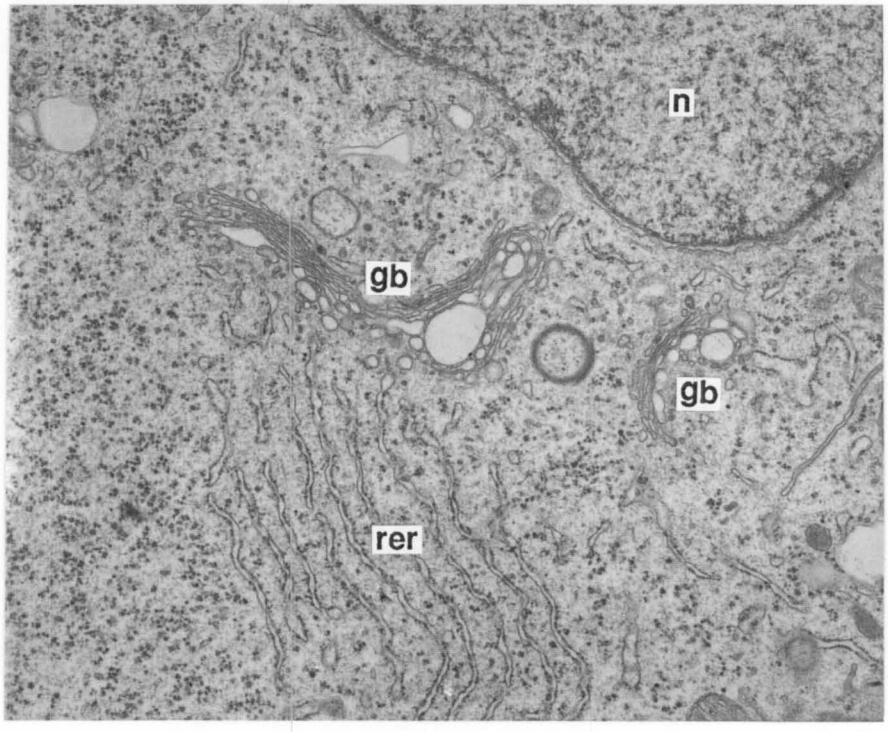


Figure 2.29 High power view of the deep basal infoldings (**bi**) of an epithelial flange cell which considerably increase the membrane surface area of the cell. Mitochondria (**m**) are packed in between.



500 nm

Figure 2.30 Abundant rosettes of glycogen (**gl**) and mitochondria (**m**) in the basal region of an epithelial flange cell adjoining a lacuna (**la**).



500 nm

Figure 2.31 Golgi bodies (**gb**) and rough endoplasmic reticulum (**rer**) close to the nucleus (**n**) in a gill epithelial cell.

suggested that the spines may serve a mechanical function in keeping the filaments apart in muddy water and help reduce the collapse of the filaments when a crayfish leaves the water (Swain *et al.*, 1988).

Taylor and Taylor (1992) state that in the freshwater crayfish the efferent branchial vessel is larger in diameter than the afferent branchial vessel, but the transverse sections did not show such an obvious difference. The movement of the haemolymph within a filament is from the afferent vessel to the efferent vessel via the lateral lacunae which follow the circumference of the filament and has been reported for the crayfish *Astacus pallipes* (Fisher, 1972), *Astacus leptodactylus* (Dunel-Erb *et al.*, 1982), *Jasus novaehollandiae* (Rogers, 1982) and *Procambarus clarkii* (Burggren *et al.*, 1974). The vascular casts of the lacunae of *Astacus pallipes* and *A. leptodactylus* indicate that the lacunae are "labyrinthine conduits" wrapped about the afferent and efferent vessels for the length of the filament (Dunel-Erb *et al.*, 1982).

The water ventilating the gills passes the length of the gill and the filament which lies parallel to the gill stem. This results in a composite co-current/cross-current/counter-current gas exchange system. The afferent vessel would be co-current, and the least efficient, the haemolymph passing through the lacunae would be cross-current to the water, and the return haemolymph flow in the efferent vessel would be counter-current, and the most efficient. This three step process could still result in very efficient exchange at the filament surface.

The transverse sections indicate that there are two different types of filament. In one the external cuticle is 1.2 µm thick and lined with a layer of epithelial tissue 7 - 10 µm thick and epithelial flange cells or pear cells (Taylor and Taylor, 1992) up to 35 µm deep. The other filament type has a cuticle 0.7 µm thick and a thin epithelial layer, with a total haemolymph to water distance of 1.5 to 2 µm. Taylor and Taylor (1992) list the cuticle thickness and haemolymph to water distance of select Crustacea and the distances for *P. zealandicus* are among the thinnest reported.

In *P. zealandicus* the gill filament with thin cuticle has a thin epithelial layer, and even near the neck region of a flange cell there are few mitochondria and the apical leaflets are not extensive. In a filament with thick cuticle the epithelial layer near the neck region of a flange cell is packed with mitochondria, and the apical leaflets are well developed. At the base of the apical leaflets there are many small vesicles. There are also extensive basolateral infoldings and adjacent cells are joined by septate desmosomes. Large quantities of glycogen, numerous Golgi bodies and extensive rough endoplasmic reticulum are also associated with these cells and indicate a high level of metabolic activity.

Dickson *et al.* (1991) describe very similar transverse sections for the gill filaments of *Procambarus clarkii* and identified an ion transporting and a respiratory filament. They also found that no filament had both characteristics, and the preliminary results suggest that this is also the case in *P. zealandicus*. In *P. clarkii* the cuticle of the respiratory filament was 0.7 µm thick and the adjacent thin epithelium had few mitochondria and no basal infoldings or apical microvilli. The ion transporting filament had a cuticle which was 1.8 µm thick, and a thick adjacent epithelial layer with numerous mitochondria, deep basolateral infoldings and numerous microvilli (Dickson *et al.* 1991).

The association between salt absorbing function and specific morphological structures was explored in the blue crab *Callinectes sapidus* by Copeland and Fitzjarrell (1968). The crab can survive in brackish water down to 0.5 ‰ salinity (sea water ≈ 35 ‰) and the putative salt absorbing region of the gills, identified by silver staining, increased in area as the salinity in the medium decreased. The cells in this region had numerous apical microvilli, extensive infoldings of the basal surface, abundant mitochondria associated with the infolded basal membranes and the adjacent cells were connected by septate desmosomes. The endothelium in these salt absorbing regions was about 10 µm thick. The remainder of the gill was considered to be the respiratory area and the endothelium was only 0.5 µm thick. The cell bodies in the

salt absorbing region of the gills also contained many Golgi bodies, extensive rough endoplasmic reticulum, microtubules, vesicles at the ends of the apical microvilli and large quantities of granules and formed bodies (possibly glycogen).

Cioffi (1984) describes the organization of cells in a generalised transport epithelium. The cells have extensive elaboration of the cell membrane, at the apical surface with extensive microvilli, and at the basal surface with basal infoldings which penetrate deep into the cell. Adjacent cells are joined at the apical surface by septate desmosomes. The cells are packed with mitochondria, Golgi bodies and rough endoplasmic reticulum, indicative of high levels of metabolic activity.

Dickson *et al.* (1991) used silver staining to identify the putative ion exchange regions on the gills of *P. clarkii*, and they measured the concentration of Na,K-ATPase and the trans-epithelial potentials (TEP) in the filaments of stained and unstained regions. They found that the filaments which had dense silver stain also had high levels of Na,K-ATPase, a high TEP, a thick cuticle and thick epithelium, extensive elaboration of the basal and apical cell membranes, many mitochondria, Golgi bodies and extensive rough endoplasmic reticulum. These gills were considered to be associated with ion exchange. The gill filaments which showed no silver staining had low levels of Na,K-ATPase, a low TEP, a thin cuticle and epithelium, minimal elaboration of the basal and apical cell membranes, and few mitochondria, and were considered to be dedicated to respiration.

Morse *et al.* (1970) found pear cells with numerous mitochondria, extensive basolateral infolding, and numerous microvilli at the apical region in *Pacifastacus leniusculus*. The ion transporting cells of *Astacus leptodactylus* are described in the same way by Bielawski (1971). Felder *et al.* (1986) consider the abundant mitochondria associated with extensive infolding of the basolateral membranes and the presence of apical microvilli with associated small vesicles as characteristics commonly associated with salt transporting tissues which are necessary for hyperosmotic regulation.

The morphological differentiation found between the

different gill filaments of *P. zealandicus* is consistent with the functions of respiration and ion-regulation being carried out in different filaments. The respiratory filament has a thin cuticle with a thin adjacent epithelium and consequently a short distance between the external medium and haemolymph. The ion-regulating filament has the thicker cuticle and thicker adjacent epithelium, apical and basal cell membranes with elaborate folds and a high density of organelles within the cells.

Conclusions.

The gill formula for *Paranephrops zealandicus* was : 20 gills, one rudimentary gill, and one epipodite. The gill filaments may terminate in a protuberance, a hook, a curved spine, two curved spines (on the epipodite), or have smooth rounded ends. The distribution of these filaments is unclear.

Two distinct gill filament types have been identified, with different internal morphology, and no evidence has been found of a gill filament altering its morphology along its length. One filament has a thin cuticle of 0.7 µm with an associated epithelium of similar thickness, no elaborate cell membranes and few organelles within the cell. The other filament type has a thick cuticle of 1.2 µm and an associated epithelium of 7 µm to 10 µm, elaborate apical and basal cell membranes and the cells are packed with organelles. The cellular differentiation suggests that the two filament types are involved in two different functions, gas exchange and ion exchange (Fisher, 1972; Rogers, 1982; Dickson *et al.*, 1991). No efforts were made to identify the relative distribution and abundance of respiratory and ion-regulating filaments. Although there were several types of external morphology in the gill filaments of *P. zealandicus*, the differences cannot be attributed to a respiratory or ion regulation function.

CHAPTER 3

SPONTANEOUS EMERSION BY *PARANEPHROPS ZEALANDICUS*

I. INTRODUCTION

In Chapter 1 the terrestrial habitats and activities of crayfish, mainly Cambarids and Parastacids, were introduced. Much of the information about the terrestrial behaviour is concerned with the habitat of the crayfish. This includes the studies by Hobbs (1981) and Horwitz and Richardson (1986) into the nature of crayfish burrows and the amount of water in these burrows.

Hobbs (1981) describes burrows on the basis of the burrowing behaviour of the crayfish with:-

"Primary burrowers" being, in general, solitary crayfish which spend almost their entire lives below the surface of the ground in complex burrows which are rarely connected with open water.

"Secondary burrowers" being crayfish which spend most of their lives in burrows but in the rainy season when the water table rises the animals will move into open water.

"Tertiary burrowers" being animals which live in open water and only construct burrows when gravid or during droughts.

Horwitz and Richardson (1986) on the other hand describe burrows in relation to the water table regardless of the resident species and they describe types:-

1a) in permanent water, the phreatic zone.

1b) connected to permanent water, the phreatic zone.

2) connected to the water table, the epiphreatic zone.

3) independent of the water table, the vadose zone.

In both systems there are situations where the animal must be an air-breather. The question which arises is - why

are some of these animals so very terrestrial, what forces drove them out of the water initially and ultimately produced such a dramatic change in habit and habitat?

There is considerable evidence, anecdotal and from researchers, which indicates that crayfish are found out of the water and often some considerable distance from free water, and that some recent crayfish populations can only have been established by terrestrial migration. Huxley (1896 in Taylor and Wheatly, 1980), Williams and Hynes (1976), Taylor and Wheatly (1980), Brinck (1983), McMahon and Wilkes (1983), Taylor and Tyler-Jones (1985), Hogger (1988), Jones and Morgan (1994), all write of crayfish emerging from water in response to hypoxia, food shortage, population density or for no known reason at all.

In their field guide to Australian Crustacea, Jones and Morgan (1994) mention that the Lamington Plateau crayfish *Euastacus sulcatus* is sometimes seen walking through the forest considerable distances from the nearest stream. Some Australian crayfish have been found in artificial ponds kilometres from the nearest crayfish population (Hogger, 1988), and this suggests they are able to tolerate not only the physiological stress of inhabiting temporary inland water bodies but also the stress of terrestrial migration.

The terrestrial habit of many of the Parastacoidea has been discussed by several authors (Hogger, 1988; Hobbs, 1988; Horwitz and Richardson, 1986), but little has been said of *Paranephrops*. Jones in his studies of *Paranephrops* growth (Jones, 1981a) and aquaculture potential (Jones, 1981b, 1985), and Hopkins in his extensive studies of *Paranephrops* growth, ageing and population structure (Hopkins, 1966, 1967a, 1967b) and systematics (Hopkins, 1970) make no reference to any form of terrestrial habit. The papers written about *Paranephrops* physiology (Wong and Freeman, 1976a, 1976b, 1976c) or ecology (Chilton, 1913; Scott and Duncan, 1967; Carpenter, 1977; Musgrove, 1988a, 1988b) make no mention of any form of escape response or emergence from the aquatic environment.

Aerial excursion by *P. zealandicus* has been reported in

the Christchurch Press by Hillary (1989) writing about a Southland (New Zealand) tourist walk, and reported to me by Mr Shane Ashcroft (Reefton) and Mr Lance Thomas (Timaru) who have been breeding and rearing *Paranephrops* in an attempt to establish an aquaculture industry. The aquaculturists have observed mass voluntary emersion at night when crayfish populations in the holding ponds are very high. The emerged crayfish proved to be very vulnerable to predation by the common hedgehog *Erinaceus europaeus* and the morning would reveal the remains of the feast (L. Thomas, *pers. comm.*).

Because the reports were limited and inconclusive it was decided to investigate whether *Paranephrops zealandicus* left the water voluntarily. The crayfish would be put into one end of a tank, Figure 3.1, which is divided into two water bodies connected by sloping surfaces, and the level of activity and degree of emersion would be recorded over a period of at least 72 hours. The experimental conditions would be varied to determine if emersion by the crayfish is influenced by the conditions in the water. Quilter (1975) and Devcich (1979) have observed diel activity patterns in both *P. zealandicus* and *P. planifrons*, and this may also be reflected in emersion behaviour recorded in this experiment.

II. MATERIALS AND METHODS

(1) Collection and maintenance of animals

Crayfish of both sexes were collected from a little stream feeding Lake Georgina. All ovigerous females were immediately returned to the stream to maintain the stream population and all animals with missing chelae were returned to the stream to reduce unnecessary losses through aggression and cannibalism once the animals were confined in holding tanks. The animals were transferred to the aquarium room at the Zoology Department where they were kept in 70 cm x 40 cm x 50 cm deep tanks which were supplied with a low flow of fresh bore water. The animals in each tank had a surplus of refuges made from plastic tubing of varying lengths and diameters. The aquarium room and the tanks were maintained at 15 ± 1°C under a 12 hour day 12 hour night light cycle.

The experimental animals were kept on an *ad lib.* feeding regime, and were not starved before the trials as the search for food may be an additional pressure to emerge from the water. They were judged to be at the intermoult stage (stage C) of the moult cycle. As emersion has not been reported to be related to weight this was not selected for. Selection of a specific weight class may result in emersion not being observed if there is a weight specific response. The animals used weighed between 15 g and 61 g, and can all be considered to be sexually mature crayfish (Hopkins, 1967a).

(2) Materials

The experiment required an environment in which the crayfish could be kept undisturbed. The crayfish needed to be able to leave the water easily and the activity of the crayfish had to be easily monitored and recorded. The environment also required a refuge from the experimental conditions and thus provide the crayfish with a choice.

For this experiment a tank constructed entirely of glass was used (Fig. 3.1). The tank was 1500 mm long, 200 mm wide and 350 mm deep, and was divided into two bodies of water.

The experimental space was a wedge of water approximately 700 mm long, 190 mm wide and 130 mm deep at the deep end. At the shallow end of this wedge the surface of the water was crossed by two weak light beams. A third light beam crossed at the line of the water's edge and a fourth beam crossed the exposed slope. A dull light beam was produced by operating 240 volt 15 Watt lamps at 120 volts. The four light beams were approximately 75 mm apart and 5 mm in diameter. Four sensitive Darlington transistors connected to four channels of a seven channel amplifier registered any break of the light beams. The amplifier also produced a range of time signals. The output from the four light sensors and a 30 minute time signal were recorded on an eight channel Rustrak Recorder with the chart advancing at a rate of 15 cm/24 hours (1 inch/4 hours).

The experimental area of the tank had a gentle slope of 3:16 (approx. 10.6°). A steep slope of 45° connected this to the second body of water at the other end of the tank. The

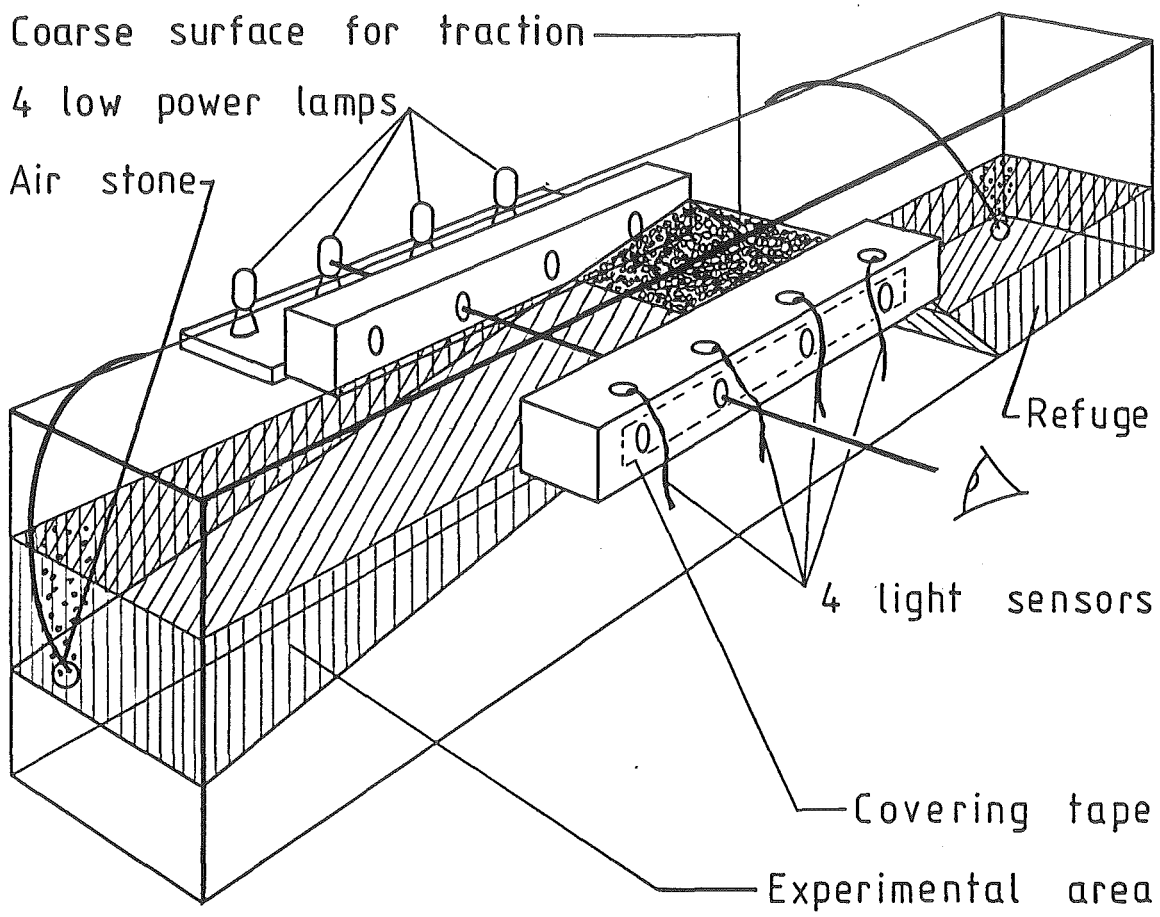


Figure 3.1 Sketch of the experimental tank (1.5 m long, 0.2 m wide, 0.35 m deep) with all covering removed. The hatched regions are the two water bodies, the experimental space on the left and on the right the "refuge".

second water body was considerably deeper, 450 mm long, and was a potential refuge from the experimental conditions. Both slopes had coarse surfaces for traction. Several large rocks provided some cover in the refuge but there was no cover in the experimental area.

The tank was covered on all sides with black paper and the top was covered with heavy card with a 6 cm × 20 cm opening over the refuge to provide diffuse light and daily cues (for dusk and dawn). During the initial trials a low-light video camera was mounted over the refuge and the cover over the refuge was removed late every evening to enable all activity down the experimental slope to be recorded. Light for the camera was provided by a distant low power red lamp.

(3) Experimental regimes

Crayfish activity was recorded for four different treatments. They were:-

a) solitary crayfish at 18°C in oxygenated water,
b) a pair of crayfish at 18°C in oxygenated water,
c) solitary crayfish at 18°C in water with reducing oxygen levels,
d) solitary crayfish at 24°C in oxygenated water.

Each trial was run for a minimum of 72 hours, and each animal was used only once. Crayfish of similar sizes were used in the treatment requiring paired animals. Hypoxia was produced in the experimental area by bubbling nitrogen through the water; however great difficulty was experienced in reducing the oxygen partial pressure as there was a large surface area available for re-oxygenation of the water. This was overcome by covering the water with a single layer of expanded-styrene beads. The PO_2 in the water was regularly monitored with an oxygen electrode attached to a Radiometer PHM71 Mk2 Acid-Base Analyzer. In the 24°C treatment the water temperature was maintained with a fish-tank heater and thermostat. The refuge was at 18°C for all the trials. The water in the refuge was kept oxygenated at all times and all the water in the tank was changed after each trial to remove the potential of contaminated water influencing the outcome.

Crayfish living in streams spend the daytime hidden in burrows, under rocks and logs or deep within weed beds, and are not often seen. They will only be subjected to diffuse light, and must use this to indicate the end of the day. The 6 cm × 20 cm opening in the heavy card cover above the refuge provided the light necessary to maintain daily cues, and this was controlled to provide a fixed day length of 12 hours.

(3) Data analysis and statistical methods

Each trial was run for a minimum of 72 hours. When a crayfish broke a light beam a mark was made by the Rustrak recorder, and these were added up in half-hour intervals for each of the four light beams.

The difference between each sensor level and the four

treatments was analyzed using oneway Anova. Bartlett's test for homogeneity of variances indicated significant differences ($P < 0.0001$). The data were re-analyzed with the Kruskal-Wallis Nonparametric Anova test and Dunn's Multiple Comparison Test.

The difference in activity levels, between daytime and nighttime, was analyzed for each treatment. All data within a treatment was divided into 24 hour segments (maximum of 2 per 72 hour trial). Trials where deaths or experimenter disturbance had occurred were excluded. In a 24 hour segment the nighttime activity recorded by all four sensors was summed. Similarly in a 24 hour segment the daytime activity recorded by all four sensors was summed. The differences (night - day), from all 24 hour segments within a treatment, were compared with a one sample two tailed "t" test to determine if they were significantly different from zero.

The data analysis was done with the InStat version 2.04 computer package from GraphPad Software. All results are tested at the 5% level of significance, and data are presented as the mean ± 1 standard error of the mean.

III. RESULTS

(1) Behaviour

The use of the low-light video camera confirmed that the events recorded by the Rustrak recorder were due to crayfish activity. The crayfish were observed exploring the shallow water and then proceeding up the slope on one or other side of the tank, not the centre, to emerge from the water. A brief period of grooming type behaviour was common when the animals first emerged. The crayfish were observed moving down the slope into the refuge and returning back up the slope into the experimental area. The animals did not appear to have any difficulty climbing the steep slope into and out of the refuge.

(2) Experimental results

Table 3.1 provides the mean ± sem. per crayfish, for the

Table 3.1 The average activity recorded per crayfish at each sensor over a period of 72 hours for the four different treatments. Sensors 1 and 2 are over the water, sensor 3 is at the water's edge and sensor 4 is over the slope and out of the water.

Experimental treatment		Mean	± sem	Grp	Significant comparisons	# 4th Sensor	## In Refuge
	1st Sensor	10.47	2.1	A	J* L*	11	4
Solo	2nd Sensor	7.68	1.88	B			
crayfish	3rd Sensor	4.74	1.15	C			
	4th Sensor	2.47	0.67	D			
N = 19	Weight g	33.78	2.17				
	Temp. °C	17.99	0.68				
	1st Sensor	9.45	2.36	E		18	14
Crayfish	2nd Sensor	12.65	3.68	F	J** K* L*		
pairs	3rd Sensor	14.4	3.82	G	J** K* L*		
	4th Sensor	9.9	3.19	H			
N = 20	Weight g	33.59	2.52				
pairs	Temp. °C	18.16	0.56				
	1st Sensor	7.35	1.46	I	J* L*	9	3
Hypoxic	2nd Sensor	1.35	0.54	J	A* F** G** I*		
water	3rd Sensor	2.15	1.04	K	F* G*		
	4th Sensor	2.15	0.95	L	A* F* G* I*		
N = 20	Weight g	30.19	1.52				
4 died	Temp. °C	17.82	0.3				
	1st Sensor	6.69	3.12	M		6	0
Water	2nd Sensor	3.31	1.4	N			
at 24°C	3rd Sensor	13.75	8.38	O			
	4th Sensor	10.12	7.03	P			
N = 16	Weight g	31.89	2.18				
	Temp. °C	24.19	0.32				

Kruskal-Wallis Nonparametric Anova Statistic KW = 61.468, P < 0.0001.
Significant results * = P<0.05, ** = P<0.01, from Dunn's multiple comparison test.
Number of trials in which a crayfish crossed the fourth sensor.
Number of individuals found in the refuge at the end of the experiment.

number of events recorded over the experimental period of 72 hours at each light sensor for all the trials in each of the four treatments. The Kruskal-Wallis Nonparametric Anova produced a significant outcome (P < 0.0001). The results are also compared as treatments within sensor level in the histogram in Figure 3.2.

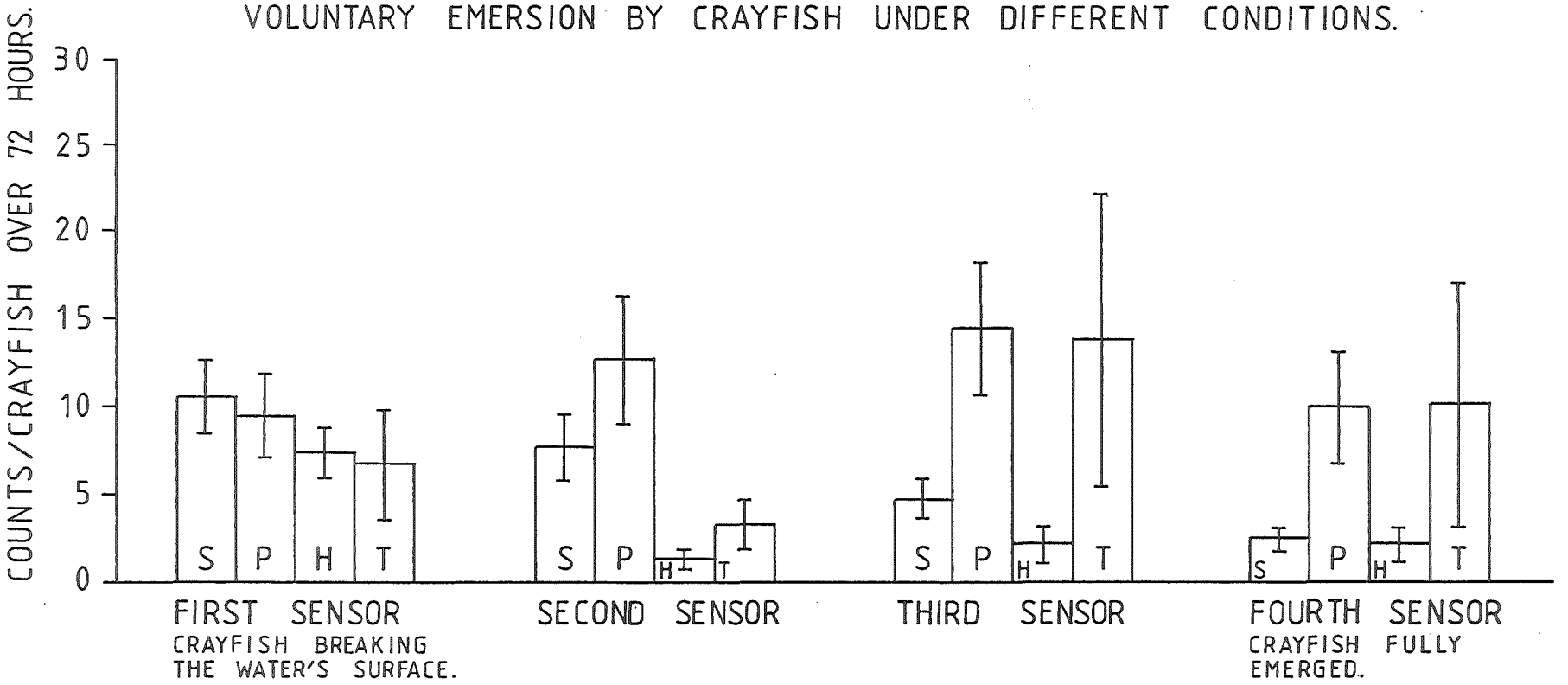


Figure 3.2 Histograms of the number of times a crayfish crossed a light beam over a period of 72 hours for the four different treatments and the four sensors. Sensor 1 and 2 are over the water, sensor 3 is at the edge of the water and sensor 4 is over the dry surface. **S** = solo crayfish, n = 19, **P** = crayfish pairs, n = 20 pairs, **H** = hypoxic conditions, n = 20, **T** = temperature elevated to 24°C, n = 16. The significant differences are indicated in Table 3.1. Data is presented as the mean ± 1 sem.

The solo crayfish recorded decreasing levels of activity as they moved up the slope and out of the water. The paired crayfish, in contrast, recorded high levels of activity per crayfish, at all positions up the slope, with the highest level recorded at the third sensor at the water's edge. The crayfish subjected to hypoxia were most active at the first sensor, and recorded very little activity at the other three sensors. At 24°C the crayfish recorded more activity out of the water at sensors 3 and 4 than at sensors 1 and 2. The Kruskal-Wallis Nonparametric Anova test produced a $P < 0.0001$ and Dunn's multiple comparisons indicate that there are some significant differences (Table 3.1).

Table 3.1 also indicates how many times an animal was found in the refuge at the end of a trial, and the number of trials in which an animal recorded activity at the fourth sensor. In the treatment with paired animals, 12 trials ended with an animal in the refuge. In 8 trials the lighter animal was displaced, in 2 trials both animals were found in the refuge, in 1 trial the lighter male displaced the heavier female, and in 1 trial the lighter male displaced the heavier male. The lighter animals were 91.1% ± 9.3% the weight of the heavier animal with a range of 64.3% to 99.6%. A Chi-squared test for homogeneity ($P = 0.05$) indicated that there was no significant difference between the treatments in the number of animals found in the refuge at the end of the trials, and in the number of trials in which activity was recorded at the fourth sensor. It was observed that in the experiment with crayfish pairs the displaced animal remained in the refuge, in the other experiments the animals did not.

In the treatment involving hypoxia the PO_2 fell to a third of ambient in 12 hours, was below 40 torr by 24 hours, and by day three the mean value was almost 30 torr, Table 3.2 and Figure 3.3. The condition of the crayfish was carefully monitored but in four of the trials the crayfish died. Of these four, two had only crossed the first sensor (trials H8 and H9), one had proceeded as far as the third sensor at the water's edge (trial H6), and the last had been very active and crossed the fourth sensor seven times (trial H17).

Crayfish of all sizes from 15 g to 61 g emerged from the

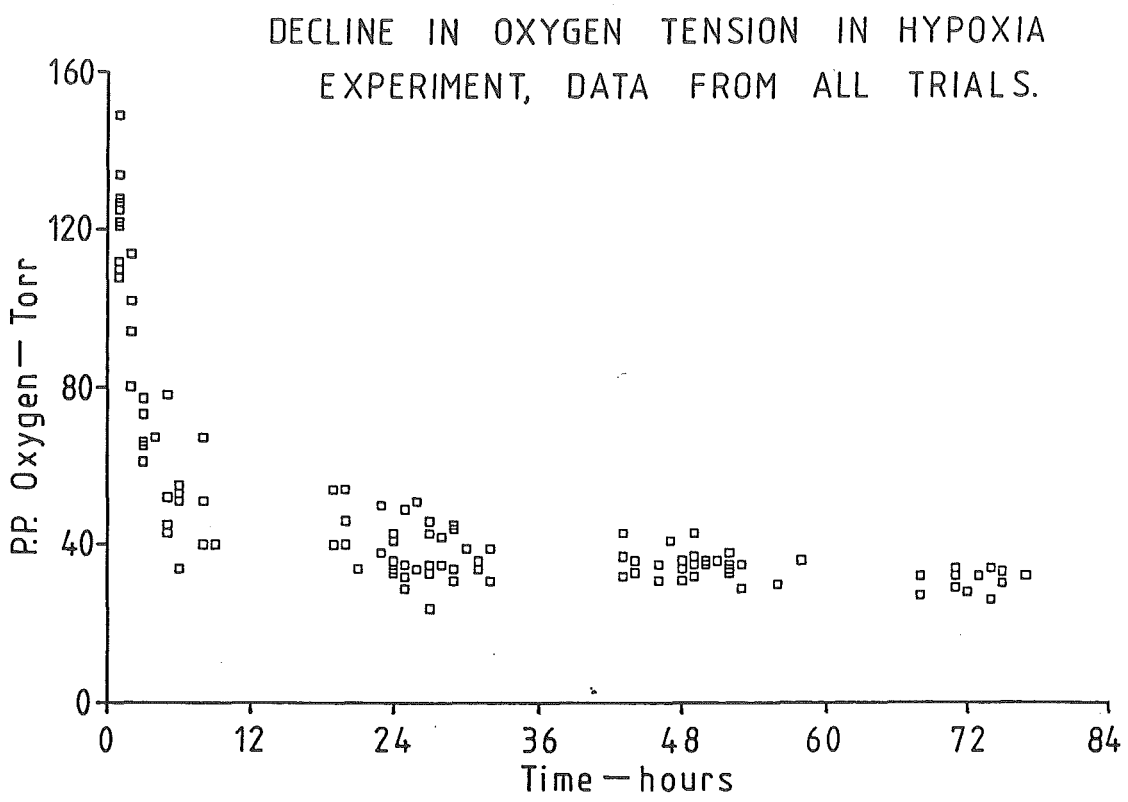


Figure 3.3 Scatter plot of the decline in PO_2 taken from all the trials over the 72 hour duration of the experiments.

water, and both active and inactive crayfish could be found at all sizes. This great individual variation in activity has been reported by Quilter (1975) in a study of circadian rhythms in *P. zealandicus*. This individual variation in activity resulted in considerable variance when emersion activity was analyzed. Figures 3.4, A to D, illustrate emersion activity recorded in four trials, a solo crayfish at 18°C, a solo crayfish at 24°C and two trials with crayfish pairs.

Table 3.2 Summary of measurements of the decline in aquatic PO_2 in the treatment exposing crayfish to hypoxia.

Sample Time, h.	n =	PO_2 (torr) Mean	± Stdev.
1 - 9	38	88.34	34.8
19 - 32	37	38.68	7.2
43 - 58	26	35.12	3.5
68 - 77	13	30.85	2.6

Analysis of the emersion activity in the four treatments indicated significantly more emersion activity at night by solo crayfish at 18°C ($t = 3.702$, 23 df, $P = 0.0012$) and

crayfish pairs at 18°C ($t = 3.247$, 35 df, $P = 0.0026$), and no significant differences in crayfish in hypoxic water at 18°C ($t = 1.103$, 31 df, $P = 0.2786$) or solo crayfish in water at 24°C ($t = 1.444$, 31 df, $P = 0.1586$).

Figure 3.5 summarises the 24 hour activity patterns not evident in the data summaries. The 24 hour plots are an average taken from the sum, over 3 consecutive 24 hour periods, of all activities recorded by the four sensors in all the trials in a treatment. The graphs show low levels of activity by the crayfish subjected to hypoxia and solo crayfish in oxygenated water, both at 18°C. Crayfish experiencing an elevated water temperature were more active in the early morning, but during the day their activity was at a low level similar to the other 2 treatments. Paired crayfish showed the highest level of activity, with peaks at 3 am and 7 am, and with low levels of activity from noon to early evening.

IV. DISCUSSION

From these experiments it is very clear that *Paranephrops zealandicus* leaves the water spontaneously, and voluntarily exposes its respiratory system to the risks and rigours of aerial respiration.

There are no significant differences in emersion activity between the solo crayfish at 18°C and solo crayfish at 24°C. There are significant differences between crayfish pairs and the crayfish subjected to hypoxia at the level of the 2nd and 3rd sensors; the crayfish experiencing hypoxia demonstrating the lower level of emersion activity. There is a confounding factor; the crayfish experiencing progressive hypoxia have a layer of styrene beads covering the water to minimise oxygen exchange. The presence of crayfish in the refuge indicates that this did not inhibit emersion in all animals, but the death of four crayfish suggests that at least this number did not resort to aerial respiration to ensure survival. This is surprising, especially as two of the four crayfish had emerged to the water's edge during the trial. If the styrene

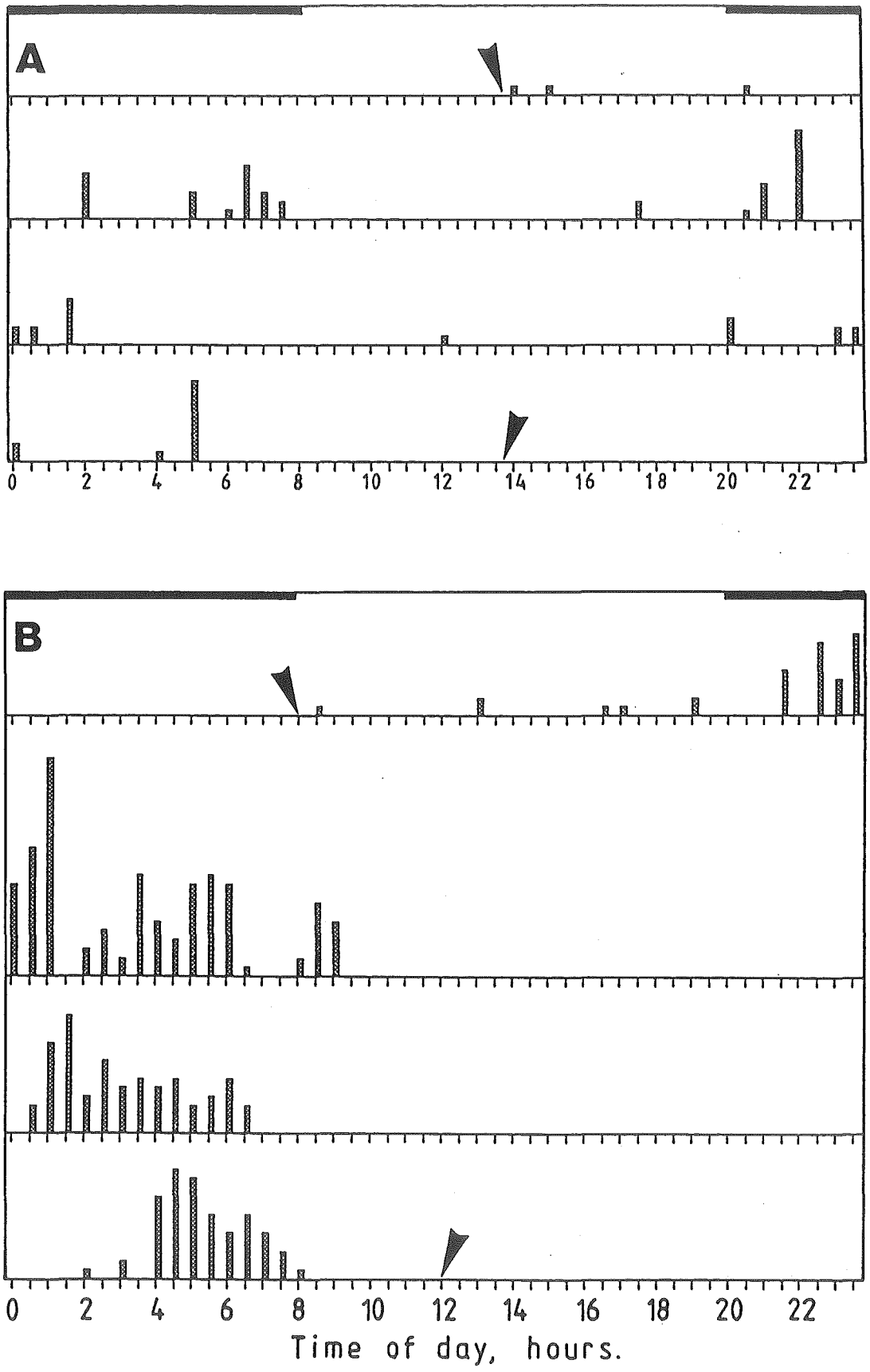
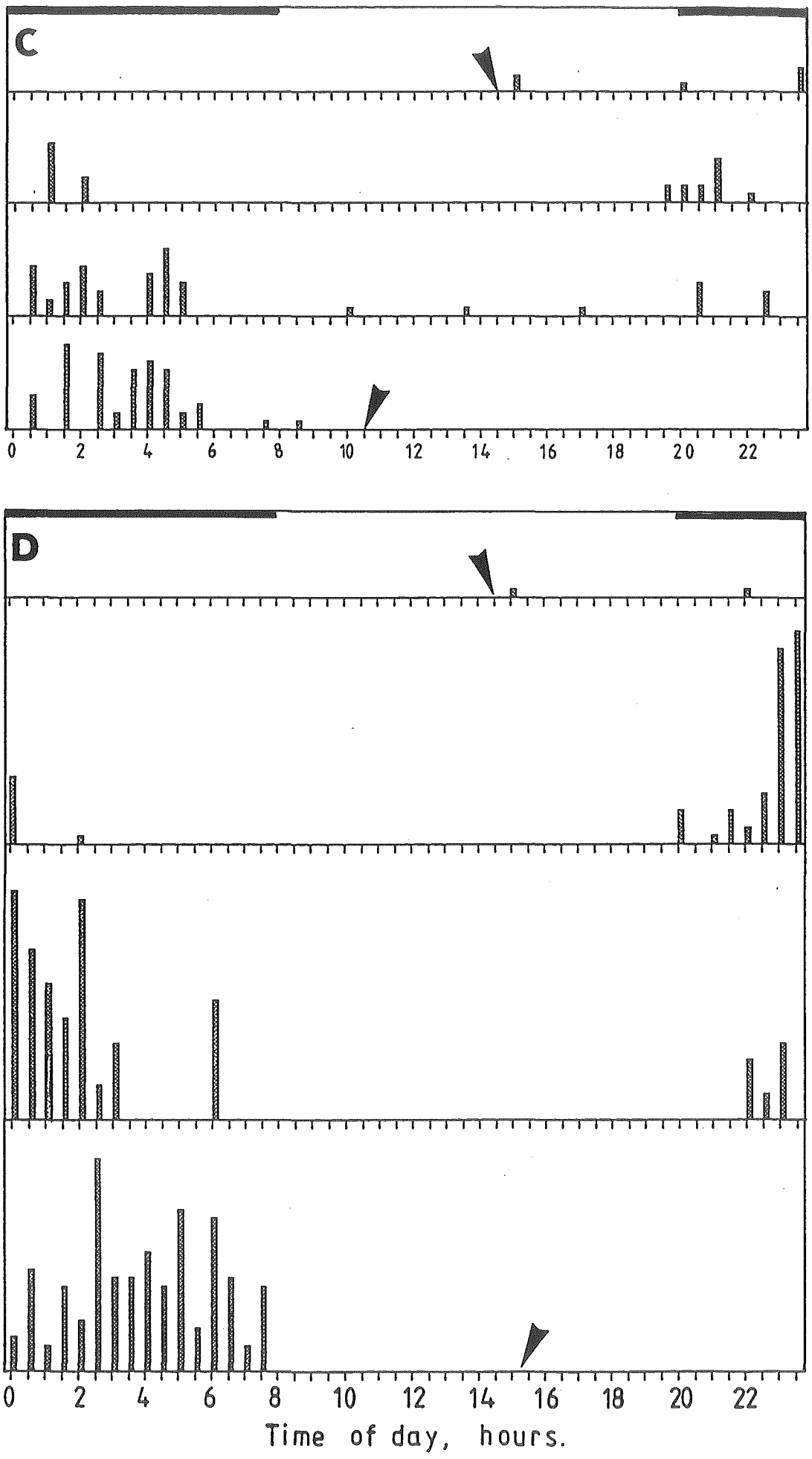


Figure 3.4 Crayfish emersion activity recorded by the four sensors. The pointers indicate the beginning and end of the trials, and the black bars at the top of each figure indicate darkness. A is from a solo crayfish at 18°C (trial 15) and B is from a solo crayfish at 24°C (trial T4). C and D on the next page are of the emersion activity recorded from crayfish pairs (trials 25 and 41).


C
D
0
2
4
6
8
10
12
14
16
18
20
22
Time of day, hours.

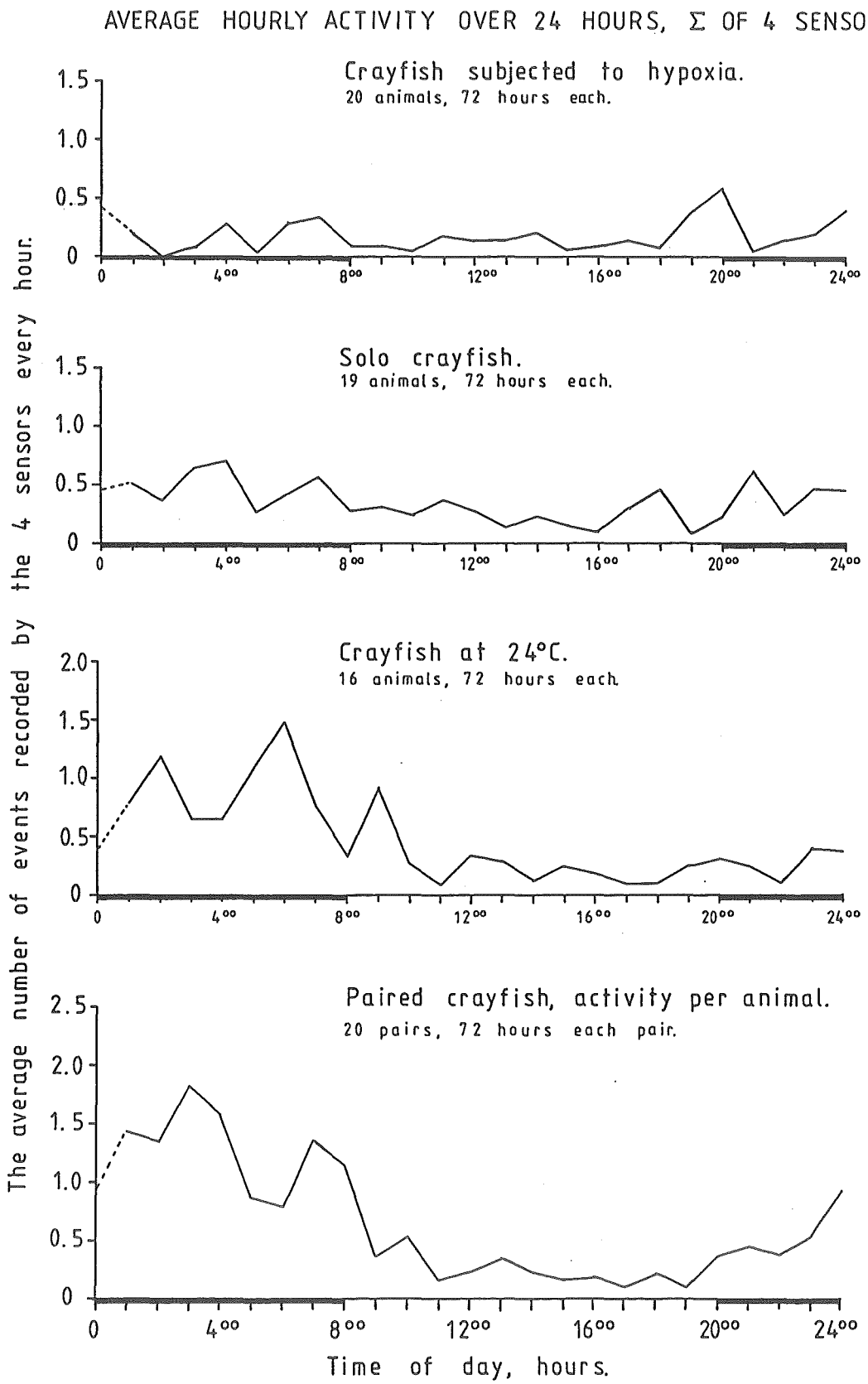


Figure 3.5 Average hourly activity per crayfish over 24 hours for four different treatments. The graphs represent the activity recorded at the water's edge, both in and out of the water, and is averaged from the sum of the data collected for all animals, over 72 hours, and four sensors. The black bars at the bottom of the figure indicate darkness.

beads did inhibit crayfish emersion then the difference in emersion activity between the crayfish experiencing hypoxia and the crayfish pairs is likely to disappear, resulting in no differences between all four treatments.

Hogger (1988) suggests that emigration of crayfish from an established population would generally only occur when the holding capacity of the habitat was exceeded, and mentions that one of the principal factors governing the size of a population is the availability of a suitable habitat and for a non-burrowing species this means suitable refugia. The other major influence is food supply. *Procambarus clarkii* are observed in large numbers migrating overland and across roadways to find new sources of water when field draining begins for the crayfish harvest (Sommer and Goldman, 1983). In their study of aggression and the effects of food and shelter, Capelli and Hamilton (1984) found that excess food did not reduce aggressive interactions to the same extent as adequate shelter. *Astacus astacus* would leave the water at night to graze the verge of the ponds when the populations were dense (Brinck, 1983), and *Austropotamobius pallipes* emerges in search of food (Huxley, 1896, in Taylor and Wheatly, 1980).

In eight encounters the lighter animal was displaced to the refuge and Capelli and Hamilton (1984) suggest that size was the single most important factor governing the outcome of an aggressive interaction between crayfish, with even a small advantage usually resulting in dominance. In most animals aggression generally increases as important resources become scarce and cannibalism seems to be a common response to both density and nutrient stresses. After predation had been accounted for, cannibalism and the quality of the artificial food supplies were the major causes for mortality in high density populations (Pursiainen *et al.*, 1983; Goddard, 1988) was particularly significant in populations with surplus adult males (Goddard, 1988), and is also considered a major source of mortality in the dense populations of crayfish farming (Sommer and Goldman, 1983).

In the non-burrowing crayfish, are refugia required for protection from cannibalism in times of food shortage, ie

predation by conspecifics, or for protection from other predators, for even a burrow in times of food shortage is no guarantee of protection from conspecifics and emigration may be a common choice. The lack of a significant difference in the number of animals in the refuge at the end of the trial with the crayfish pairs suggests that two crayfish may not be a critical density in this situation. The tendency for the displaced animal to remain in the refuge indicates that the avoidance of aggressive encounters may be important.

The animals subjected to hypoxia had the lowest levels of activity. As indicated in Table 3.2, the P_{O_2} in the water was below 40 Torr after one day, and went down to 30 Torr. For *P. zealandicus* this is below P_{CRIT}, which lies between 41 and 44 Torr (Chapter 6), and is the P_{O_2} at which oxygen consumption becomes compromised. At 15°C the crayfish *A. pallipes* left the water and remained in the air when the P_{O_2} in the water dropped below 42 ± 5 torr (Taylor and Wheatly, 1980), and when aquatic P_{O_2} levels were maintained below 30 torr *Orconectes rusticus* were frequently seen emerging temporarily into air (McMahon and Wilkes, 1983), where they trapped air in their branchial chambers which on resubmergence was released periodically as bubbles. *Carcinus maenas* exploits the availability of oxygen in the aerial environment in response to hypoxia (Taylor, Butler and Sherlock, 1973; Wheatly and Taylor, 1979), and (Wheatly and Taylor, 1979) suggest that the animal retains the aquatic route for CO_2 exchange by aerating the water surrounding the gills. *Parastacoides tasmanicus* leaves hypoxic water in preference to continued immersion, and in extremely hypoxic conditions ($P_{O_2} \leq 17$ torr) the animal uses a well-developed emigration response involving a regular cycle of immersion and partial exposure, gaining access to aerial oxygen, using the water for CO_2 removal, and avoiding potential acid-base changes in the haemolymph (Swain, Marker and Richardson, 1987).

P. zealandicus appeared to remain in the water and did not use the aerial oxygen supply, even when P_{O_2} in the water was below the level necessary to maintain $\dot{M}O_2$. This must ultimately lead to problems as the use of anaerobic energy to

supplement energy requirements produces lactic acid, and too much lactic acid with the consequent changes in haemolymph pH can result in death. This was potentially the fate of the four crayfish which died during the experiment.

In disposing of CO_2 water breathing creatures have a distinct advantage over air breathing creatures. Because water has a very high capacity for CO_2 it is easily removed at the gills so that water breathers have low levels of CO_2 dissolved in their haemolymph. Although hypoxia can be avoided by emergence, this produces an inevitable rise in haemolymph PCO_2 and with it potentially dangerous changes in haemolymph chemistry occur. One stratagem to avoid this is to use the oxygen in the air and retain the aquatic route for CO_2 excretion, as mentioned for *P. tasmanicus* and *C. maenas* above. The very low levels of emersion activity recorded by *P. zealandicus* subjected to hypoxic water suggests that this is not being used. However, as previously mentioned, the low level of activity may be attributable to the presence of the styrene beads on the surface of the water, so this outcome needs to be considered with caution.

The animals subjected to an elevated temperature are very active at the water's edge and further up the slope, and may experience some evaporative cooling out of the water at the potential risk of desiccation. *Austropotamobius pallipes* was able to survive up to 3 days at 15°C and 70-80% r.h. (Taylor and Tyler-Jones, 1985), and this provides some scope to emerge and remain cool till water temperatures drop.

At 18°C, solo crayfish and crayfish pairs recorded a higher level of activity in the shallow water and out of the water at night than during the daytime. Solo crayfish at 24°C show distinct increases in activity during the hours of darkness (Figure 3.5), but the individual variation in activity results in a non-significant result.

Crayfish subjected to hypoxia were so inactive in the shallow water and out of the water that it was not possible to identify a significant difference in activity between day and night. This lack of activity has already been discussed, and consequently the outcome needs to be treated with caution. The response of other Crustacea to hypoxia suggests

that *P. zealandicus* is a likely candidate for an increase in emersion activity as oxygen becomes less available, not the almost total suppression of emersion activity which was recorded.

Orconectes nais has a low metabolic rate through the day which increases after darkness to a peak during the night (Rice and Armitage, 1974), *Orconectes clypeatus* has a 24 hour rhythm of locomotor activity and oxygen consumption with peak activity during the night (Fingerman and Lago, 1957), and *Orconectes rusticus* was involved in aggressive encounters more often at night (Capelli and Hamilton, 1984). Activity and trappability studies of *Astacus astacus* and *Pacifastacus leniusculus*, and SCUBA studies in their lake habitats, indicated that both species were dark-active throughout the year, both species were most active about midnight, and temperature influenced the level of activity (Abrahamsson, 1983). *In the water b*oth *Paranephrops* species have been found to be more active at night (Quilter, 1975; Devcich, 1979), and the results indicate that this increase may also extend to emersion.

Conclusions.

It is clear that *Paranephrops zealandicus* leaves the water voluntarily and spontaneously.

Emersion activity data for the crayfish experiencing hypoxia needs to be treated with caution, the other three treatments do not demonstrate significant differences in the degree of emersion activity.

At 18°C, solitary crayfish and crayfish pairs show higher levels of emersion activity at night than during the daytime.

CHAPTER 4

OXYGEN CONSUMPTION IN WATER AND IN AIR

I. INTRODUCTION

Crayfish, with some exceptions, are aquatic animals, and in water their gills have near neutral buoyancy. The tissues of the gills are very thin and fluid pressure is an important structural element helping keep the gills spread out and functioning. When the high-shore crab *Cyclograpsus lavauxi* was removed from the water and the residual water drained from the branchial chambers the gill lamellae were drawn into bundles by water surface tension (Innes *et al.*, 1986). This is a common problem in bimodal crabs (O'Mahony and Full, 1984; deFur, 1988) and the delicate filaments of a crayfish gill do not fare any better (Taylor and Wheatly, 1980). Emersion and the subsequent loss of water from the branchial chamber may cause clumping of the gills, reducing the surface area available for respiration. The resistance to gas diffusion may also be increased by the film of water covering the gills increasing the effective thickness of the barrier between the air and the haemolymph.

Emersion may affect Astacoidea and Parastacoidea differently because of structural differences in the design of the gills. Astacoidea have epipodites associated with the podobranchs, (Chapter 2). These are absent from the gills of the Parastacoidea which have one epipodite, attached to the first maxilliped. The epipodites, while possibly conferring an advantage in aquatic respiration by directing the flow of the water over the gills in the most efficient manner, have the potential to disadvantage the crayfish in the air by enveloping the gills and keeping the water trapped, limiting the effective area available for gas exchange. The Parastacoidea, not having epipodites on the podobranchs, may be able to shed water from the gills more rapidly and

completely, and this may enable a more rapid and complete adjustment to aerial respiration.

When first emerged, a crayfish may have to maintain oxygen consumption through a reduced gill area and with an increased resistance to gas diffusion until sufficient trapped water is lost for the gill elements to separate. An initial increase in resistance to diffusion would result in haemolymph oxygen being consumed faster than it is replenished, producing in a drop in haemolymph PO_2. As the diffusion rate is dependent on the gradient between the air and the haemolymph, once haemolymph PO_2 is low enough the increase in diffusion rate may again make it possible to meet oxygen needs.

If the crayfish is not able to obtain enough oxygen to maintain itself out of the water then it must reduce its metabolism or supplement its needs with anaerobic metabolism. Anaerobic metabolism produces potentially toxic end products which will further limit the ability of the animal to survive out of the water. Therefore any possible limitations which the gills may impose on aerial respiration may be reflected in the ability of an aquatic animal to maintain its oxygen consumption in air.

Taylor and Wheatly (1980, 1981) found that the crayfish *Austropotamobius pallipes* kept in air for 24 hours maintained its $\dot{M}O_2$ at the same level as settled submerged animals, and when the animal was returned to water the $\dot{M}O_2$ rose briefly and then settled to normal within 8 hours. The recovery time and elevation of $\dot{M}O_2$ after 24 hours in air was of a similar magnitude to that seen in *A. pallipes* recovering from only 3 hours exposure to air, and similar to the recovery pattern in water recorded after handling (Taylor and Wheatly, 1980). According to Taylor and Wheatly (1981), this behaviour suggested that there was not a progressive accumulation of an oxygen debt. The increased $\dot{M}O_2$ may have been to replenish the depleted venous reserve (Taylor and Wheatly, 1980) or caused by the stress and disturbance associated with the return to water (Taylor and Wheatly, 1981). The depletion of the venous reserve is caused by the drop in haemolymph P_aO_2 and P_vO_2 experienced during aerial respiration, as will be seen

in Chapter 8, which results in a low haemolymph oxygen content (Taylor and Wheatly, 1980).

Terrestrial Crustacea, or those which are bimodal breathers, such as *Carcinus maenas* (Taylor and Butler, 1978), *Cardisoma guanhumi* and *Gecarcinus lateralis* (O'Mahoney and Full, 1984), *Holthuisana transversa* (Greenaway, Bonaventura and Taylor, 1983; Greenaway, Taylor and Bonaventura, 1983), *Heloecius cordiformis* (Maitland, 1990), *Cyclograpsus lavauxi* (Innes *et al.*, 1986), and *Austropotamobius pallipes* (Taylor and Wheatly, 1980), appear to be able to maintain oxygen consumption in air at levels similar to their oxygen consumption in water. To date there has not been a detailed study of oxygen consumption in air, or the effects of aerial respiration, in any of the Parastacoidea.

Fingerman and Lago (1957) found a 24 hour rhythm in locomotor activity and oxygen consumption in *Orconectes clypeatus* and Rice and Armitage (1974) found a daily rhythm in the metabolic rate of *Orconectes nais*, both species demonstrating peak activity during the night. In water both *Paranephrops* species have also been found to be more active at night (Quilter, 1975; Devcich, 1979), but there is no available information to indicate that this may extend to the metabolism and respiration of a crayfish out of the water.

The experiments presented in this chapter measure the rate of oxygen consumption of the crayfish *P. zealandicus* both in water and for an extended period in air, and will establish if the crayfish is able to maintain its oxygen consumption for an extended period out of the water. Subsequent experiments (Chapters 5 & 8) will measure the haemolymph PO_2, to determine if there has been a change in the diffusion gradient between the respiratory medium and the haemolymph. The measurements will be from settled animals, for if a settled animal is not able to maintain its oxygen consumption adequately then the aerial environment will only be accessible for brief periods, and not at all available for major excursions or extended periods away from water.

II MATERIALS AND METHODS

(1) Collection of animals

Crayfish of both sexes were collected from a little stream flowing into Lake Georgina. Ovigerous females and animals with missing chelae were returned to the stream. The animals were kept in 70 cm x 40 cm x 50 cm deep tanks in the aquarium room at the Zoology Department. This is maintained at 15 ± 1°C with a 12 hour day 12 hour night light cycle. The tanks were supplied with running bore water and each tank had surplus refuges made from plastic tubing. The animals were not fed for 48 hours before they were used for the experiment; they weighed between 9 g and 61 g and were judged to be at the intermoult stage (stage C) of the moult cycle.

(2) Experimental Methods

The oxygen consumption of Crustacea in water may be measured in several ways. The animal may be fitted with a mask so that both branchial flow and oxygen consumption can be measured (Larimer, 1961; Arudpragasam and Naylor, 1964; Hughes *et al.*, 1969; Taylor, 1976; McMahon *et al.*, 1979; Greenaway *et al.*, 1983; O'Mahoney and Full, 1984). Oxygen consumption can be measured from a continuous water flow through the respirometer (Taylor *et al.*, 1977a, 1977b; Butler *et al.*, 1978; Taylor and Wheatly, 1979, 1980, 1981; Wheatly and Taylor, 1981; Swain *et al.*, 1987; Wheatly, 1989) and also in a closed respirometer (Sutcliffe *et al.*, 1975; Sutcliffe and Carrick, 1975; Bridges and Brand, 1980; Houlihan *et al.*, 1984; Houlihan and Innes, 1984; and Innes *et al.*, 1986).

Each method has its difficulties. Measurements may be influenced by the stress of wearing the mask and also by the tendency of many Crustacea to reverse ventilate. An open respirometer requires the measurement of the flow rate and the oxygen content of both input and outflow water, and a closed respirometer requires thorough mixing of the water if reliable results are to be achieved. A mask and an open respirometer do not allow easy measurement of aerial oxygen consumption and are not easily switched between aquatic and aerial respirometry. Closed respirometry is the simplest,

and may easily be applied to several animals simultaneously. A respirometer for both aquatic and aerial oxygen consumption in which the animal experiences the change from water to air without handling and minimal disturbance is described in Titulaer (1991). The respirometer, of which six were built, is a perspex tube which is clamped between two end-plates each with an opening for inlet or exhaust of water or air. The tube has openings to be used for sampling, draining, and introducing CO_2 absorbing materials.

In a closed respirometer good mixing of the water is crucial to obtain good results as the oxygen consumption in water is calculated from the measured fall in PO_2 over time. Dye was introduced into a 270 ml respirometer with a syringe and a clock and mixing motor were started simultaneously. Movement of the dye in the respirometer was recorded with a sequence of photographs. A 49 g crayfish was put into the respirometer and the process was repeated. Figure 4.1 shows the rate of dye movement through the respirometer. In the empty respirometer total mixing of the dye occurred within 30 seconds. The crayfish reduced the initial acceleration of the water but by 15 seconds the water had travelled further than in the empty respirometer, as there was less water to move, and total mixing was well within 30 seconds. Rapid movement of the water within the respirometer ensures more rapid and thorough mixing of the water and means that changes in PO_2 are more quickly registered.

The oxygen consumption during aerial respiration was measured with a manometer in the manner of Gilson (1963) and Davies (1966). Aerobic metabolism uses oxygen and produces CO_2, and in a closed respirometer, if the CO_2 produced by respiration is absorbed by chemicals, then the consumption of oxygen will result in a drop in pressure. Constant pressure, which is measured by the manometer, is maintained by reducing the volume of the air in the respirometer, and this drop in air volume is a measure of the oxygen consumed (Umbreit *et al.*, 1972).

Because of the anticipated oxygen consumption, based on other Crustacea and the size of the experimental animals, and the need to measure aquatic and aerial oxygen consumption, a

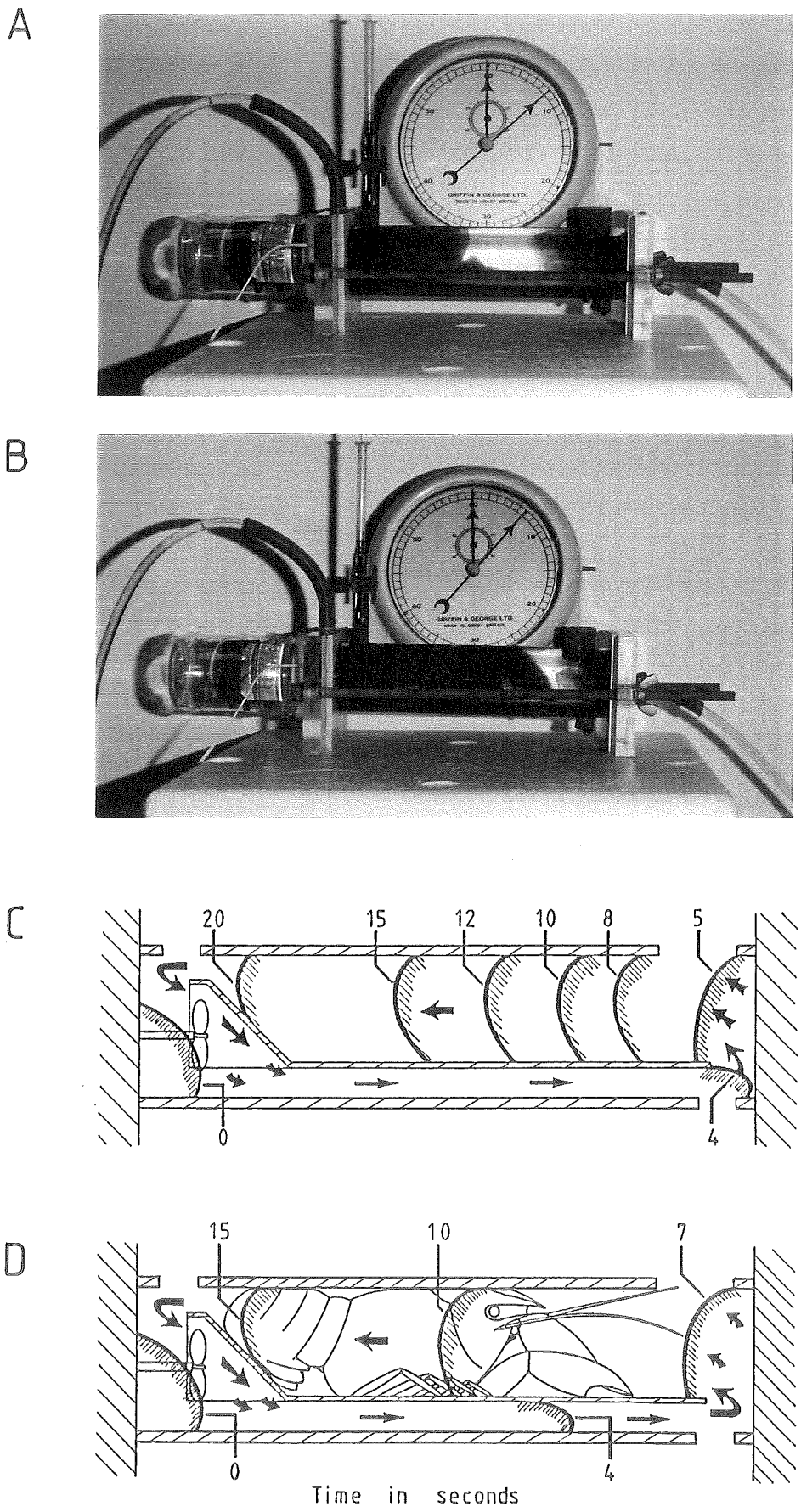


Figure 4.1 Dye flow in a respirometer, A after 8 seconds, and B with a crayfish after 7 seconds, and sketches of dye flow in a respirometer, C empty, and D with a crayfish.

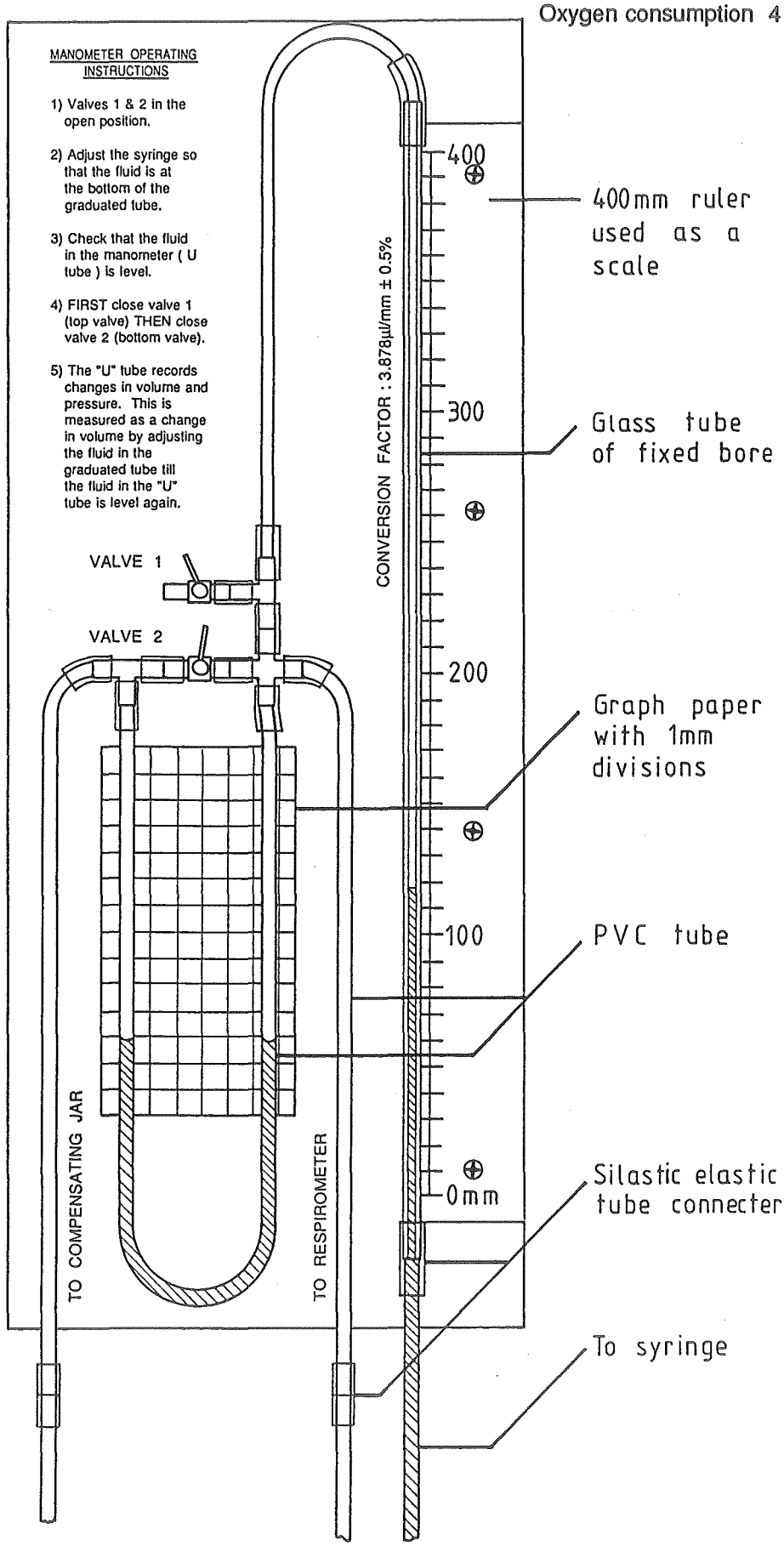


Figure 4.2 Sketch of the manometer built from components bought from a tropical aquarium fish shop. For larger volumes Davidson (1994) replaced the 1.5 ml graduated tube with a 5 ml graduated pipette.

manometer was built which was able to accommodate large air displacements and was independent of the respirometer (Figure 4.2). The PVC tubing, valves, "T" and "X" junctions are standard items used for air supply for hobby fish tanks, and the 400 mm ruler used as a scale is a standard school item. The only special item is the glass tube which is a calibration tube of fixed bore and is commonly used in physics/chemistry laboratories. The tube was calibrated by filling the capillary with distilled water with the tube on a sensitive balance. The calibration in µl volume per mm length was then calculated and the accuracy was calculated from the variability of the six tubes employed for the six manometers which were built.

Umbreit *et al.* (1972) discuss the problems of using potentially permeable plastic tubing in a manometer and considers that this causes no problem if the composition of the gases within respirometer differs little from the composition of the surrounding air. Provided the total length of tubing out of the water bath is the same on both sides of the manometer, then small temperature variations in the laboratory should have negligible effect on the accuracy of the results.

The water bath could accommodate five respirometers. Four respirometers had crayfish in them, and a blank control was used for microbial oxygen consumption. Each respirometer including the blank was darkened with black polythene sheet to prevent the movements of the experimenter disturbing the animals. All five respirometers were connected to a common manifold which was supplied with recirculating oxygenated water from the water bath or with humidified air, and this made the change from aquatic to aerial respiration very simple.

The crayfish were weighed and put in the respirometer in a water bath at 15°C ± 0.2°C. During aquatic respiration the respirometers had a sampling syringe in one opening and in another opening a 5 ml syringe without a plunger and full of water, compensating for the sample loss. To measure $\dot{M}O_2$ the respirometer was closed off with clamps on the incoming and exhaust water lines. At regular intervals water samples were

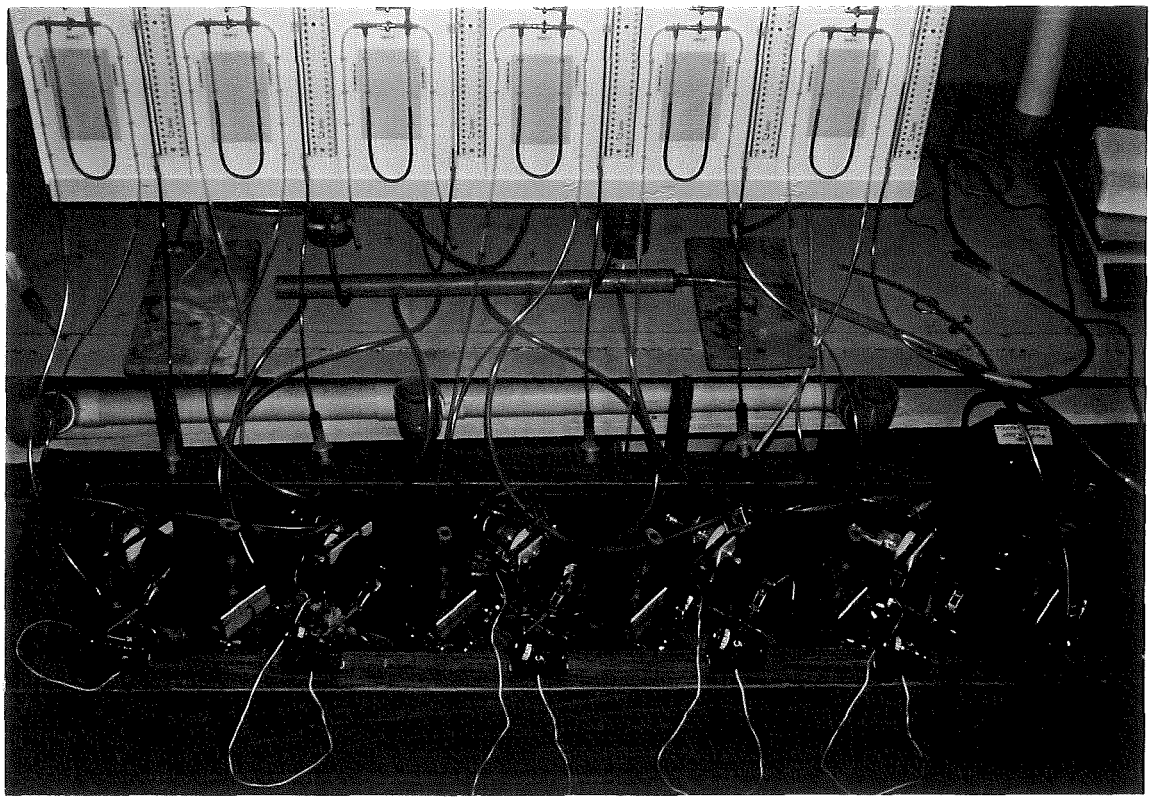


Figure 4.3 Five respirometers in the water bath connected to five manometers on the bench above with the air/water manifold lying on the bench below the manometers. There is one spare manometer in case of breakdown.

taken for PO_2 measurement until there were sufficient data to calculate the $\dot{M}O_2$. The PO_2 was measured by a Strathkelvin 1302 oxygen electrode connected to a Strathkelvin model 781B oxygen meter. As the water samples were 0.5 ml or less the addition of less than 0.2% volume fresh water (0.5 ml in 270 ml) is not considered significant.

Using undisturbed Crustacea at comparable or higher temperatures, respiratory independence of P_IO_2 has been demonstrated down to 60 Torr in *Carcinus maenas* (Taylor, 1976), 51 Torr in *Cherax tenuimanus* (Villarreal, 1990), 40 Torr in *Astacopsis franklinii*, and 30 Torr in *Parastacoides tasmanicus* (Swain *et al.*, 1987). The measurements in this experiment were stopped and the oxygenated water supply was reconnected to the respirometer after one hour or if the PO_2 in the respirometer fell below 80 Torr. The change from aquatic to aerial respiration was achieved by switching the pump which circulated water through the respirometer off, and turning the air supply on, driving the water out of the

lowest port in the respirometer.

To measure the $\dot{M}O_2$ in air the respirometer was closed, the manometer replaced the sampling syringe, and CO_2 absorbing material, Sodasorb, in a small container firmly attached to a rubber bung, was introduced through the opening which had been used for the compensating syringe. The graduated tube on the manometer was able to handle a volume of 1500 µl before readjusting, a process which took about 10 seconds, but which was rarely needed. The air supply was restored after sufficient measurements had been taken to calculate the $\dot{M}O_2$, a process which usually took less than an hour. At the end of 48 hours aerial respiration the process was reversed to restore the crayfish to aquatic respiration and to monitor recovery.

(3) Experimental protocol

Taylor and Wheatly (1980) found that the crayfish *Austropotamobius pallipes*, in water, settled within five hours after a disturbance with an oxygen consumption not significantly different from that observed after 18 hours settling. Preliminary trials conducted over 24 hours indicated that *P. zealandicus* achieved settled $\dot{M}O_2$ in water within eight hours.

The experimental conditions were:-

Settling - which was started between 10.00 am and 11.00 am. $\dot{M}O_2$ was measured at the beginning, and after 3 and 8 hours, samples 1, 2, and 3. The final settling $\dot{M}O_2$ was measured between 6.00 pm and 7.00 pm. The respirometers were then prepared for aerial respiration.

Emersion - which was achieved by draining the water from the respirometers. $\dot{M}O_2$ was measured at the beginning, and after 3, 12, 24, 36 and 48 hours, samples 4, 5, 6, 7, 8, and 9. Emersion was started as close to 8.00 pm as possible to ensure that samples 4, 6, 7, 8 and 9 were measured as near to 8.00 am and 8.00 pm as possible. After the final aerial $\dot{M}O_2$ measurements had been taken the respirometers were filled with water for recovery measurements.

Recovery - was achieved by filling the respirometers with water and resubmerging the crayfish at 10.00 pm after the last aerial $\dot{M}O_2$ had been measured between 8.00 pm and 9.00 pm. Recovery in water was monitored, and $\dot{M}O_2$ measured at the beginning and after 3 and 10 hours, samples 10, 11 and 12.

This is similar to the study by Taylor and Wheatly (1981) on the effect of long term aerial exposure on the respiration of the freshwater crayfish *Austropotamobius pallipes*. The experiment was carried out 12 times, with 48 animals. The oxygen consumption of 46 crayfish was measured, with two of the trials abandoned, one for leaks and one for an escapee.

Paranephrops are more active at night (Quilter, 1975; Devcich, 1979), and the activity rhythm is maintained by the light/dark cycle Quilter (1975). The crayfish will only be subjected to diffuse light during the daytime when they are hiding in burrows, under rocks and logs or deep within weed beds. In this experiment the laboratory experienced natural illumination, and the polythene covers did not extend to the ends of the respirometers, allowing light through to maintain daily cues.

(4) Calculations

Oxygen consumption ($\dot{M}O_2$) by crayfish in water, in $\mu mol.g^{-1}.h^{-1}$,

$$\dot{M}O_2 = \frac{(a \times V \times \Delta PO_2 \times 60)}{(w \times t)}$$

a is the oxygen capacitance of water in $\mu mol.l^{-1}.Torr^{-1}$, 2.0101 $\mu mol.l^{-1}.Torr^{-1}$ at 15°C (Dejours, 1981, p227),
V is the volume of the respirometer in litres,
ΔPO_2 is the change in oxygen partial pressure in Torr,
w is the crayfish weight in g,
t is the duration in minutes.

Oxygen consumption ($\dot{M}O_2$) by crayfish in air, in $\mu mol.g^{-1}.h^{-1}$,

$$\dot{M}O_2 = \frac{(Q \times L \times 60 \times ß \times 273)}{(w \times t \times 760 \times °K \times 22.414)}$$

Q is the manometer conversion factor, 3.878 $\mu l.mm^{-1}$,
L manometer scale reading in mm,
ß barometric pressure on the day in Torr (mm Hg),
°K is the temperature in °Kelvin,
w is the crayfish weight in g,
t is the duration in minutes,
22.414 $l.mol^{-1}$ converts gas volume to moles at STPD, (Dejours, 1981, p229).

(5) Data analysis and statistical methods

The oxygen consumption was calculated for every crayfish at each sampling time, while settling in water, in air and during recovery, and oneway Anova with Tukey-Kramer Multiple Comparisons Test was used to identify differences between the sampling times.

Linear regressions of log oxygen consumption against log mass were calculated for all crayfish in the dataset at sample points 3 and 12 for crayfish in water and sample point 7 for crayfish in air. The difference between daytime and nighttime $\dot{M}O_2$ of crayfish out of the water was analyzed from morning/evening (8 am/8 pm) sample pairs 6/7 and 8/9 from each crayfish, and the differences (night - day) were compared with a one sample two tailed "t" test to determine if they were significantly different from zero. The $\dot{M}O_2$ of crayfish after 8 hours settling in water and after 10 hours recovering from aerial respiration, samples 3 and 12, were each compared with the $\dot{M}O_2$ of crayfish at P_{CRIT}, from Chapter 6, with an unpaired "t" test.

The data was analyzed with the InStat version 2.04 computer package from GraphPad Software. Results are tested at the 5% level of significance and the data are presented as the mean ± 1 standard error of the mean.

III RESULTS

The oxygen consumption ($\dot{M}O_2$) of crayfish while settling in water for 8 hours, samples 1, 2 and 3, exposed to aerial respiration for 48 hours, samples 4 to 9, and recovering in water for 10 hours, samples 10, 11 and 12, is summarised in Table 4.1 and Figure 4.4. The Anova produced a significant result, $F_{(11,486)} = 10.786$, $P < 0.0001$.

After three hours settling the $\dot{M}O_2$ was significantly different from the $\dot{M}O_2$ recorded when the crayfish were first put into the respirometer. All subsequent measurements were also significantly different from the initial $\dot{M}O_2$.

The $\dot{M}O_2$ during the 48 hours in air was not significantly different from the $\dot{M}O_2$ of 1.09 $\mu mol.g^{-1}.h^{-1}$ after eight hours settling in water, with a high $\dot{M}O_2$ of 1.07 $\mu mol.g^{-1}.h^{-1}$, after 24 hours in air, and a low $\dot{M}O_2$ of 0.97 $\mu mol.g^{-1}.h^{-1}$, after 36 hours in air. None of the six values for $\dot{M}O_2$ in air were significantly different from each other.

When the crayfish were returned to aquatic respiration, the $\dot{M}O_2$ was initially elevated, but still significantly lower than the initial disturbed value. During subsequent recovery in water the crayfish did not show an $\dot{M}O_2$ much different from the $\dot{M}O_2$ recorded during the later stages of settling, with the $\dot{M}O_2$ of samples 11 and 12 being similar samples 2 and 3.

The oxygen consumed during eight hours settling in water, measured from the area under the graph, is 10.7 $\mu mol.g^{-1}$, with a mean $\dot{M}O_2$ of 1.34 $\mu mol.g^{-1}.h^{-1}$, and the oxygen consumed during the first eight hours recovering in water is 10.2 $\mu mol.g^{-1}$, with a mean $\dot{M}O_2$ of 1.28 $\mu mol.g^{-1}.h^{-1}$. The oxygen consumed during ten hours recovering in water is 12.5 $\mu mol.g^{-1}$, with a mean $\dot{M}O_2$ of 1.25 $\mu mol.g^{-1}.h^{-1}$.

There was no significant difference between the $\dot{M}O_2$ of crayfish at P_{CRIT} (Chapter 6), and the $\dot{M}O_2$ of crayfish after 8 hours settling in water ($t = 0.3095$, 61 df, $P = 0.7580$) and crayfish after 10 hours recovering from aerial respiration ($t = 0.6070$, 61 df, $P = 0.5461$).

Analysis of the crayfish $\dot{M}O_2$ during the 48 hours emersion indicated no significant difference in $\dot{M}O_2$ between morning and evening (8 am/8 pm), ($t = 1.276$, 69 df, $P = 0.2062$).

Table 4.1 Table of oxygen consumption by *Paranephrops zealandicus* settling in water, 48 hours in air and recovering in water, with sample numbers of significant pairwise comparisons in the column "p < 0.05".

Summary of crayfish oxygen consumption corrected for blanks
A total of 46 animals were used, weight ± stdev 25.33 ± 10.8 g.

	sample no.	time hr	n	$\dot{M}O_2$ µmol/g.hr	stdev	S E M	p < 0.05
Settling	1	0	46	1.7031	0.4831	0.0712	2 to 12
in	2	3	46	1.3497	0.4121	0.0608	1,5,6,8
water	3	8	46	1.0923	0.336	0.0495	1
	4	9	31	1.0341	0.5224	0.0938	1
Breathing	5	12	35	1.0275	0.3445	0.0582	1,2
air	6	21	36	1.0381	0.5299	0.0883	1,2
for	7	33	39	1.0712	0.3869	0.062	1
48 hours	8	45	41	0.9753	0.357	0.0558	1,2,10,11
	9	57	40	1.0632	0.4819	0.0762	1
Recovery	10	58	46	1.3397	0.4617	0.0681	1,8
in	11	61	46	1.3228	0.4545	0.067	1,8
water	12	68	46	1.1122	0.2892	0.0426	1

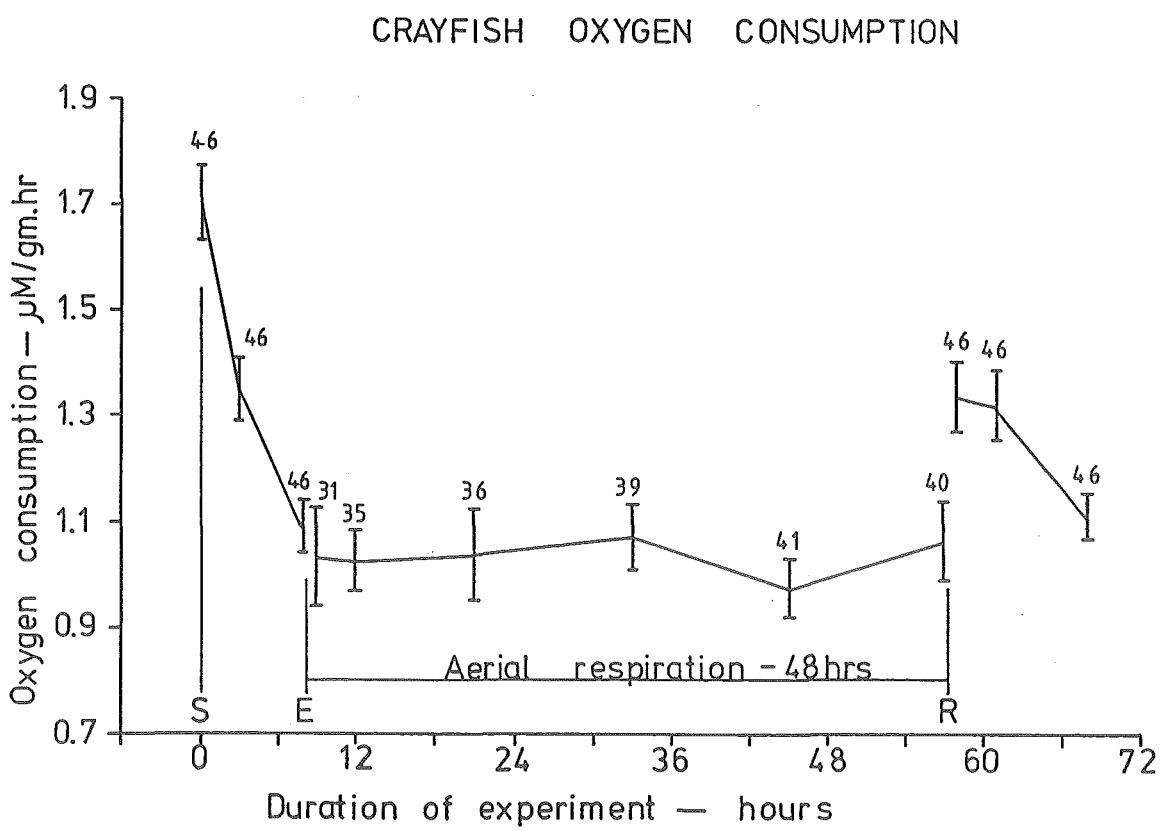


Figure 4.4 Oxygen consumption by *P. zealandicus* during 8 hours settling in water, 48 hours in air and subsequent 10 hours recovery in water. The n values are shown and the error bars represent ± 1 sem.

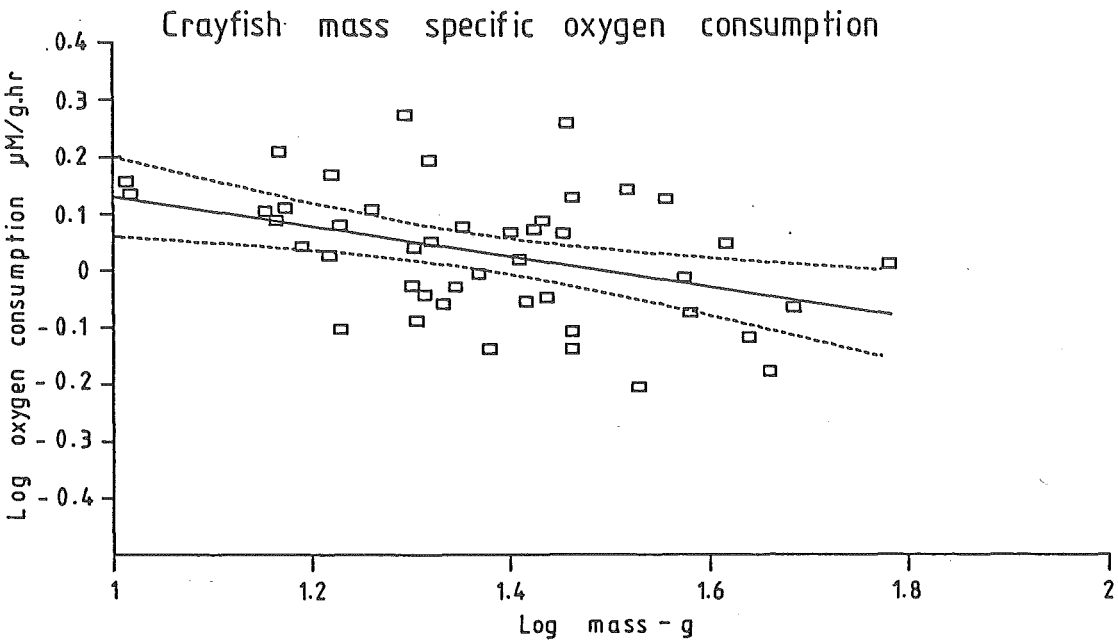


Figure 4.5 Log/log plot of mass specific oxygen consumption in water from 46 crayfish 9 g to 61 g, after 10 hours recovery from aerial respiration, sample 12, with the regression line and 95% confidence intervals.

Analysis of the $\dot{M}O_2$ difference between samples 8 and 9 alone, indicated no significant difference in $\dot{M}O_2$ between morning and evening ($t = 1.692$, 36 df, $P = 0.0993$).

A log/log regression line of mass specific oxygen consumption, with 95% confidence interval, for sample point 12, 10 hours recovering in water after 48 hours in air, can be seen in Figure 4.5. The three regression lines are;

sample 3, 46 crayfish, settled in water for 8 hours,

$\log_{10}\dot{M}O2 = 0.4141 - 0.2898 \log_{10}$mass, the slope is significantly different from zero $F = 7.677$, $P = 0.0082$, correlation coefficient (r) = -0.3854, $r^2 = 0.1486$.

sample 7, 39 crayfish, 24 hours in air,

$\log_{10}\dot{M}O2 = 0.4960 - 0.3609 \log_{10}$mass, the slope is significantly different from zero $F = 7.796$, $P = 0.0082$, correlation coefficient (r) = -0.4172, $r^2 = 0.1740$.

sample 12, 46 crayfish, 10 hours recovering in water after 48 hours in air,

$\log_{10}\dot{M}O2 = 0.3932 - 0.2642 \log_{10}$mass, the slope is significantly different from zero $F = 9.590$, $P = 0.0034$, correlation coefficient (r) = -0.4230, $r^2 = 0.1789$.

IV DISCUSSION

In Crustacea the stress of being handled or disturbed causes immediate increases in heart and scaphognathite rates, and increasing oxygen consumption (McMahon *et al.*, 1974; Butler *et al.*, 1978; Taylor and Wheatly, 1980, 1981; Chapter 8). The crayfish *A. pallipes* recorded an initial $\dot{M}O_2$ of 2 $\mu mol.g^{-1}.h^{-1}$ when disturbed in water, which settled to an $\dot{M}O_2$ of 0.9 $\mu mol.g^{-1}.h^{-1}$ after 18 hours (Taylor and Wheatly, 1980). *Orconectes virilis* also experienced increased respiratory activity and elevated $\dot{M}O_2$ after being disturbed, and required a long period of settling for the $\dot{M}O_2$ to decline (McMahon *et al.*, 1974).

When the crayfish were first put into the respirometer there was a 50% elevation in $\dot{M}O_2$ above levels recorded after 8 hours settling in water, and after 10 hours in water recovering from aerial respiration. The elevated $\dot{M}O_2$ is consistent with handling stress and being out of the water while being dried, weighed and put into the respirometer. Respiration was affected for a considerable time, with the mean $\dot{M}O_2$ during settling some 20% above the settled value of 1.1 $\mu mol.g^{-1}.h^{-1}$. The increase in ventilation also increased the P_aO_2 from the control value of 60 Torr in settled crayfish to almost 110 Torr (Chapter 5). This increased the oxygen content of the haemolymph from 800 $\mu mol/l$ at 60 Torr to 900 $\mu mol/l$ at 110 Torr (Chapter 7). Using the haemolymph content for *A. pallipes* of 12% of body weight (Rhodes, 1982), a 25 g crayfish would have a 3 ml haemolymph volume, and an increase in haemolymph oxygen content of 100 $\mu mol/l$ would result in an increase in oxygen content of 0.012 $\mu mol/g$ (whole weight). It would appear that the larger part of the increase in $\dot{M}O_2$ must be attributed to the ventilatory effort and any other activity initiated by the stress, as the increased haemolymph oxygen content of 0.012 $\mu mol/g$ crayfish will be quickly met by the increase in mean $\dot{M}O_2$ of nearly 0.24 $\mu mol.g^{-1}.h^{-1}$.

In response to increasing hypoxia (Chapter 6) crayfish regulated $\dot{M}O_2$ down from 1.32 $\mu mol.g^{-1}.h^{-1}$ to 1.07 $\mu mol.g^{-1}.h^{-1}$ as P_IO_2 declined from ambient (>150 Torr) to between 40 to 45 Torr. Below 40 Torr $\dot{M}O_2$ declined rapidly with P_IO_2. It must

be considered unlikely that the settled $\dot{M}O_2$ in water is lower than the lowest value of regulated $\dot{M}O_2$ of 1.07 $\mu mol.g^{-1}.h^{-1}$ in the face of declining oxygen tensions. This suggests that crayfish with an $\dot{M}O_2$ of 1.09 $\mu mol.g^{-1}.h^{-1}$ (sample 3) in a situation of normoxia are at a settled condition. This is supported by the lack of significant difference between the $\dot{M}O_2$ of settled crayfish, crayfish after 10 hours recovering, and the crayfish subjected to hypoxia.

The $\dot{M}O_2$ recorded from *P. zealandicus* during 48 hours in air is not significantly different from the settled aquatic $\dot{M}O_2$. If the gills imposed limitations on respiration in air, then the crayfish would not be able to maintain their $\dot{M}O_2$ in air at the same level as in water. An increase in diffusion resistance caused by residual water covering the gills, or a decrease in surface area when the gill filaments are drawn together by water surface tension, could be overcome by a drop in haemolymph PO_2. This would alter the diffusion gradient, and consequently the diffusion rate. When crayfish moved into the air, the haemolymph experienced a drop in PO_2 of 30 Torr (samples 3 and 4, Table 5.1). This would suggest that there was a change in conductance and that a change in the diffusion gradient was necessary to maintain the rate of diffusion and $\dot{M}O_2$.

The elevated $\dot{M}O_2$ during recovery from 48 hours emersion is of similar duration and magnitude to that observed when the animal is first settling in the respirometer. Although the initial $\dot{M}O_2$ in the recovery period was not as high as the 1.7 $\mu mol.g^{-1}.h^{-1}$ observed at the beginning of the experiment, the total oxygen consumption of 10.2 $\mu mol/g$ during eight hours recovery is similar to the oxygen consumption of 10.7 $\mu mol/g$ during eight hours settling. The high P_aO_2 of 100 Torr during recovery, compared with the 60 Torr in the control crayfish (Table 5.1) results in a higher haemolymph oxygen content. This suggests that there are similar processes at work during the initial settling and the recovery from aerial respiration, and indicates that there is no oxygen deficit brought about by the period of emersion, other than the reduction in haemolymph oxygen content, seen as a drop in haemolymph PO_2.

The crayfish *A. pallipes*, after it was disturbed in water, recorded an initial $\dot{M}O_2$ of 2 $\mu mol.g^{-1}.h^{-1}$ (Taylor and Wheatly, 1980). Crayfish kept in air for 24 hours maintained their $\dot{M}O_2$ at 0.9 $\mu mol.g^{-1}.h^{-1}$, and when they were returned to water they achieved a peak $\dot{M}O_2$ of 1.4 $\mu mol.g^{-1}.h^{-1}$ after two hours, and a settled $\dot{M}O_2$ of 0.6 $\mu mol.g^{-1}.h^{-1}$ after eight hours recovery (Taylor and Wheatly, 1981).

Herreid (1980) and Ellington (1983) discuss the almost universal phenomenon in invertebrates of an oxygen debt, observed during recovery from a period of environmental hypoxia or high energy demand. They mention three sources of the oxygen debt:-

1) depletion of the oxygen stores within the body fluids,

2) depletion of ATP and phosphagen stores, and

3) re-metabolising of anaerobic metabolic end products.

Anaerobic metabolism is employed to supplement energy needs when the tissues' energy demands exceed the capacity of the respiratory and circulatory systems to deliver oxygen. When the conditions for gas exchange are again favourable the respiratory effort often remains elevated. It is generally considered that the elevated level of oxygen consumption is associated with the repayment of an oxygen debt. The rate of recovery of depleted haemolymph oxygen stores is dependent on the call being made on the oxygen supply by normal metabolic needs and the need to restore anaerobic substrates (Herreid, 1980; Ellington, 1983).

The lack of accumulated lactate in the haemolymph at the end of 48 hours aerial respiration (Chapter 5) suggests that there was no oxygen deficit at the end of 48 hours in air. The period of elevated $\dot{M}O_2$ observed in *P. zealandicus* during recovery from aerial respiration was similar to that observed during settling in water at the beginning of this experiment. It is suggested that the increase in the $\dot{M}O_2$ and ventilation was caused by stress and this resulted in an increase in P_aO_2 and haemolymph oxygen content (Table 5.1).

Taylor and Wheatly (1980, 1981) and Wheatly and Taylor (1981) found that *A. pallipes* recovering from 24 hours aerial respiration demonstrated an elevation in $\dot{M}O_2$ of similar

magnitude and duration as a crayfish recovering from three hours exposure to air. The recovery was similar, in either case, to recovery in water after a period of disturbance and handling, and they concluded from this that *A. pallipes* under these conditions had no progressive accumulation of an oxygen debt.

Oxygen consumption per unit mass generally decreases as the mass of the animal increases, and experimentally $\dot{M}O_2$ is about proportional to mass$^{-0.25}$ (Alexander, 1971; Schmidt-Nielsen, 1983). (This slope is orthogonal to the slope of log.oxygen consumption in mol/h plotted against log.mass, which is considered proportional to mass$^{-0.75}$, see Schmidt-Nielsen, 1983). For *P. zealandicus* settled in water the exponents for mass specific oxygen consumption were -0.29 (sample 3) and -0.26 (sample 12), and -0.36 after 24 hours in air. In the crayfish *Austropotamobius pallipes* the exponents for mass specific oxygen consumption were -0.42 in water and -0.61 in air (Taylor and Wheatly, 1980).

Although there are reports of activity, metabolism and oxygen consumption rhythms in crayfish (Fingerman and Lago, 1957; Rice and Armitage, 1974; Quilter, 1975; Devcich, 1979), the studies involve crayfish in their aquatic environment. The crayfish in this experiment spent 48 hours in air and did not exhibit a significant $\dot{M}O_2$ difference between morning and evening although the difference between the last two samples (8 and 9) is greater than the whole dataset (6/7 and 8/9). It is possible that 48 hours in air is not long enough to produce a daily rhythm, the $\dot{M}O_2$ still being dominated by the stress of emersion.

P. zealandicus weighing between 9 g and 61 g had a settled aquatic $\dot{M}O_2$ of 1.1 $\mu mol.g^{-1}.h^{-1}$ at 15°C. This is similar to the 1.2 $\mu mol.g^{-1}.h^{-1}$ recorded for *Carcinus maenas* of similar size, 1.0 $\mu mol.g^{-1}.h^{-1}$ for *Homarus vulgaris* 4 to 9 times larger, 1.1 $\mu mol.g^{-1}.h^{-1}$ for *Eurytium albidigitum* of half the size, and lower than the 1.50 $\mu mol.g^{-1}.h^{-1}$ recorded for *Cherax tenuimanus* of similar size at 18°C (Table 4.2). These values for $\dot{M}O_2$ are lower than several species reported in Table 4.2, especially the $\dot{M}O_2$ of 3.7 $\mu mol.g^{-1}.h^{-1}$ for the

Table 4.2 Summary of crustacean oxygen consumption from several authors. The table indicates the normal habitat of the animal and the conditions under which oxygen consumption was measured. The abbreviation "calc." indicates that this value is one the authors of the experiment have derived from their data.

Species	Habitat	Weight g	Temp. °C	Condition	$\dot{M}O_2 \pm$ SE µmol/g.h	Source
Carcinus maenas	shore crab	17 - 101	15	water, settled water, disturbed air, settled	1.22 ± 0.067 2.21 ± 0.297 2.61 ± 0.23	Taylor and Butler (1978)
Callinectes sapidus	marine crab	88 - 147	24-26	water air	3.7 ± 0.251 1.29 ± 0.274	O'Mahoney and Full (1984)
Cardisoma guanhumi	semi-terrestrial	83 - 176	24-26	water air	2.01 ± 0.354 2.19 ± 0.417	
Gecarcinus lateralis	land crab	51 - 75	24-26	water air	0.62 ± 0.110 4.15 ± 0.607	
Pachygrapsus crassipes	intertidal	16 - 26	23	water air	1.86 ± 0.12 0.84 ± 0.12	Burnett and McMahon (1987)
Eurytium albidigitum	intertidal	22 - 33	23	water air	1.14 ± 0.18 0.054 ± 0.012	
Heloecius cordiformis	aerial crab (lungs & gills)	1.9 - 3.7	25	wet dry	4.61 ± 0.69 4.76 ± 0.60	Maitland (1990)

Table 4.2 cont.

Species	Habitat	Weight g	Temp. °C	Condition	$\dot{M}O_2 \pm$ SE µmol/g.h	Source
Leptograpsus variegatus	supralittoral	4.7 - 57	25	air	3.83 calc.	Greenaway *et al* (1992)
Cancer magister	marine crab	551 - 960	8	water	1.48 ± 0.36	McMahon *et al* (1979)
Holthuisana transversa	land crab (lungs & gills)	20 - 30	25	water	1.65	Greenaway, Bonaventura & Taylor (1983)
		11 - 31	25	air, disturbed air, settled	2.95 0.45	Greenaway, Taylor & Bonaventura (1983)
Cyclograpsus lavauxi	high-shore crab	1.5 calc.	10	water air	1.45 calc. 1.41 calc.	Innes *et al* (1986)
Homarus vulgaris	marine lobster	220 - 510	15	water	1.04 ± 0.06	Butler *et al* (1978)
Austropotamobius pallipes	f.w. crayfish	7 - 91	15	water, disturbed water, settled air	1.99 ± 0.53 0.73 calc. 0.70 calc.	Taylor and Wheatly (1980)

Table 4.2 cont.

Species	Habitat	Weight g	Temp. °C	Condition	$\dot{M}O_2 \pm$ SE µmol/g.h	Source
Pacifastacus leniusculus	f.w. crayfish	16 - 33	5	water	1.28 ± 0.24	Rutledge and Pritchard (1981)
			10	water	1.47 ± 0.32	
			15	water	1.79 ± 0.22	
			20	water	2.81 ± 0.25	
			25	water	2.57 ± 0.23	
			30	water	3.44 ± 0.28	
Astacopsis franklinii	f.w. crayfish	8.9 - 16.5	15	water	2.05 ± 0.27	Swain *et al* (1987)
Parastacoides tasmanicus	f.w. crayfish	8.8 - 15.1	15	water	1.91 ± 0.22	
Cherax tenuimanus	f.w. crayfish	13.3 - 51.5	18	water	1.50 calc.	Villarreal (1990)
			22	water	2.25 calc.	
Paranephrops zealandicus	f.w. crayfish	2 - 24	18	water	2.26 ± 0.64	Worner (1976)
Paranephrops planifrons	f.w. crayfish	1.8 - 11.5	18	water	5.56 ± 0.58	
Paranephrops zealandicus	f.w. crayfish	9 - 61	15	water	1.1 ± 0.04	This study
				air	1.03 ± 0.07	

crab *Callinectes sapidus*, and the $\dot{M}O_2$ of 5.6 $\mu mol.g^{-1}.h^{-1}$ for *P. planifrons*. The $\dot{M}O_2$ of *P. zealandicus* is 1.7 $\mu mol.g^{-1}.h^{-1}$ when it is disturbed, similar to the $\dot{M}O_2$ of 2.2 $\mu mol.g^{-1}.h^{-1}$ from *Carcinus maenas*, and 2 $\mu mol.g^{-1}.h^{-1}$ from *A. pallipes*.

P. zealandicus settled in air had an $\dot{M}O_2$ between 1.0 and 1.1 $\mu mol.g^{-1}.h^{-1}$, which was not significantly different from the settled $\dot{M}O_2$ in water. *A. pallipes* maintain their $\dot{M}O_2$ at 0.92 $\mu mol.g^{-1}.h^{-1}$ settled in water and at 0.94 $\mu mol.g^{-1}.h^{-1}$ for 24 hours in air (Taylor and Wheatly, 1980, 1981), but the $\dot{M}O_2$ declines to 0.67 $\mu mol.g^{-1}.h^{-1}$ by the end of 48 hours in air (Taylor and Wheatly, 1981). *P. zealandicus* is therefore different from *Austropotamobius pallipes* in its ability to maintain its oxygen consumption in air for longer.

P. zealandicus has a lower settled aerial $\dot{M}O_2$ than many Crustacea which inhabit the terrestrial environment. It was similar to the crayfish *A. pallipes* and the high shore crab *Cyclograpsus lavauxi*, and about half the $\dot{M}O_2$ of the shore crab *Carcinus maenas*, and the semi-terrestrial crab *Cardisoma guanhumi*. The $\dot{M}O_2$ is very different from species considered to be terrestrial such as *Gecarcinus lateralis*, with an $\dot{M}O_2$ of 4.2 $\mu mol.g^{-1}.h^{-1}$, and *Heloecius cordiformis*, with an $\dot{M}O_2$ of 4.6 $\mu mol.g^{-1}.h^{-1}$. The desert crab *Holthuisana transversa* has, in contrast, a settled $\dot{M}O_2$ which is extremely low at 0.45 $\mu mol.g^{-1}.h^{-1}$.

The results suggest that *P. zealandicus* has stressed and settled $\dot{M}O_2$ values similar to animals of comparable size.

Conclusions.

The $\dot{M}O_2$ of *P. zealandicus* during 48 hours in air was between 0.97 and 1.07 $\mu mol.g^{-1}.h^{-1}$, and was not significantly different from the $\dot{M}O_2$ of 1.1 $\mu mol.g^{-1}.h^{-1}$ settled in water.

For *P. zealandicus* the exponents for mass specific oxygen consumption were -0.26 when settled in water and -0.36 after 24 hours aerial respiration.

Crayfish out of water did not demonstrate a significant difference in $\dot{M}O_2$ between 8 am and 8 pm.

The ability of *Paranephrops zealandicus* to maintain resting oxygen consumption in air for an extended period of 48 hours is demonstrated.

Metabolism can be maintained in air without an apparent deficit which would lead to an oxygen debt, other than a fall in haemolymph oxygen content.

The results suggest that during the first 48 hours in air the gills do not restrict aerial gas exchange and compromise the resting oxygen consumption of the crayfish.

An increase in resistance to gas diffusion across the gill results in a drop in haemolymph PO_2 and depletion of the haemolymph oxygen content, which is not recovered until conditions for gas exchange are again favourable. That the recovery does not occur until the crayfish returns to the water, suggests that the resistance to diffusion remains high throughout the period of aerial respiration.

It is important for a bimodal animal to be able to move between water and air with minimal respiratory difficulty or compromise. With a settled aerial $\dot{M}O_2$ which is similar to its settled aquatic $\dot{M}O_2$, *P. zealandicus* is able to remain out of the water for up to 48 hours without a metabolic debt, sufficient for extended periods away from water.

CHAPTER 5

HAEMOLYMPH CHANGES ASSOCIATED WITH AERIAL RESPIRATION

I INTRODUCTION

In Chapter 4 the resting oxygen consumption was found to be similar in air and in water. It was suggested that in air a residual film of water over the gills would increase the resistance to diffusion. This would result in a lowering of haemolymph PO_2 to a point where an increase in the diffusion gradient would again make it possible to acquire the oxygen needed to meet metabolic requirements. If this occurs then a crayfish in air would demonstrate a reduced haemolymph PO_2 throughout the period when a steeper diffusion gradient assists oxygen transfer across the gills.

In water CO_2 is some thirty times more soluble than O_2, and this is the source of a major difference between animals which breathe air and animals which breathe water. Taylor and Innes (1988) point out that the low availability of O_2 in water results in high ventilation rates in water-breathers. This is effective hyperventilation with respect to CO_2, which is so readily disposed of in water, and consequently aquatic animals typically have a haemolymph PCO_2 in the range of 2 to 4 Torr, a few Torr above ambient, and correspondingly low levels of 4 - 10 $mmol.l^{-1}$ total carbonates (Table 5.6; Taylor and Taylor, 1992). Haemolymph PO_2, reflecting oxygen use and availability, controls the main ventilatory drive in water-breathing animals (Taylor, 1976; Dejours, 1981; McMahon and Wilkens, 1983; Taylor and Innes, 1988).

As oxygen makes up nearly 21% of air volume, things are very different for an air breathing animal. If the amount of O_2 consumed by metabolic processes produces a similar amount of CO_2, then the CO_2 content in expired air will show a rise of similar magnitude to the fall in oxygen content (Dejours, 1981). Consequently the haemolymph PCO_2 needs to be higher

than the expired air PCO_2 if an air-breathing animal is to unload CO_2 at the respiratory surface. In some vertebrate air breathers the venous PCO_2 may be as high as 40 Torr, and is a major control of ventilatory drive.

The change from aquatic to aerial respiration is accompanied by an increase in haemolymph PCO_2 which in turn alters haemolymph total carbonate content, pH and buffering. The respiratory acidosis which occurs when a water breathing animal resorts to aerial respiration is well documented and can be seen in Table 5.6.

Considerable work has been done on rhythms in crayfish activity, metabolism and haemolymph chemistry, and Sakakibara *et al.* (1987) found that haemolymph pH of *A. leptodactylus* was 0.1 units more alkaline at dusk than at dawn. It was suggested that this was linked to an increase in metabolism. *P. planifrons* (Devcich, 1979) and *P. zealandicus* (Quilter, 1975; Chapter 3) have been found to be more active at night. There is no information, however, to indicate that this rhythm extends to the haemolymph chemistry or pH of these crayfish either in water or in air.

In Chapter 4 the oxygen consumption of settled crayfish during 48 hours aerial respiration and subsequent recovery suggested that there was no oxygen debt caused by accumulated anaerobic metabolites. L-lactate, which is readily measured, is the anaerobic end-product usually observed in Crustacea in response to a shortage of oxygen brought about by exercise or environmental hypoxia. Any build-up of lactate, then, will indicate a period of anaerobic metabolism supplementing the energy requirements of the crayfish because insufficient oxygen was available (Bridges and Brand, 1980; Ellington, 1983; Booth *et al.*, 1984; Morris *et al.*, 1986c).

To observe the changes in crayfish haemolymph caused by aerial respiration, the haemolymph PO_2, PCO_2, pH, CCO_2 and [lactate] were recorded under conditions similar to those used for measuring crayfish oxygen consumption. A drop in haemolymph PO_2 would suggest that there was an increase in gill resistance to the diffusion of oxygen. An increase in haemolymph lactate would suggest that the supply of aerobic energy was not able to meet the energy demands.

II MATERIALS AND METHODS

(1) Collection of animals

Crayfish of both sexes were collected from a little stream flowing into Lake Georgina. Ovigerous females and animals with missing chelae were returned to the stream, and the rest were taken to the aquarium room at the Zoology Department. This is maintained at 15 ± 1°C with a 12 hour day 12 hour night light cycle, and the animals were kept in 70 cm x 40 cm x 50 cm deep tanks supplied with fresh bore water and surplus refuges made from plastic tubing. The animals were not fed for 48 hours before an experiment and were judged to be at the intermoult stage (stage C) of the moult cycle.

(2) Experimental protocol

To measure the effect of prolonged aerial respiration on the PO_2, PCO_2, CCO_2, pH and [lactate] of crayfish haemolymph, the animals were put through a procedure similar to that used to measure crayfish oxygen consumption in water and in air. The crayfish were put into a length of plastic tubing of similar dimensions to the respirometers used for measuring aquatic and aerial oxygen consumption (Chapter 4). Each end of the tube was covered with a coarse 1 mm open weave nylon mesh which was secured with a tight fitting plastic ring, (Figure 5.1). The tubes were laid out on a perforated tray supported on several bricks in a large tank (Figure 5.2) in the Zoology department aquarium room. There was a continuous supply of aerated bore water to the tank which was kept at 15°C ± 1°C. For aerial respiration the water was lowered, till it was below the perforated tray, by replacing the long overflow pipe by a shorter pipe. After the animals

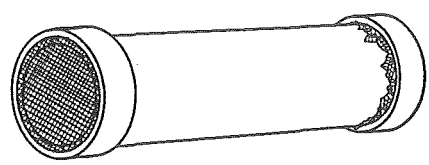


Figure 5.1 A drawing of a plastic tube with 1 mm nylon mesh on each end used to restrain the crayfish during the experiment.

had been subjected to aerial respiration for 48 hours the water level was raised, with the longer overflow pipe, and the animals were re-immersed in water. The tank was covered with a sheet of expanded styrene foam to prevent fluctuations in air temperature and to ensure the airstones were able to maintain the humidity at saturation when the crayfish were out of the water (Figure 5.2).

Crayfish were randomly removed from the tank and if haemolymph sample could not be obtained from the pericardial sinus within 20 seconds the animal was released into a recovery tank. This was to prevent the sampling of stress or activity induced changes in the haemolymph. The 0.5 ml to 0.8 ml sample was taken from the pericardial sinus with a chilled 1 ml syringe. Careful work ensured that the animals suffered little and recovered quickly. As the samples were taken from the pericardial sinus the values recorded are for oxygenated or arterial haemolymph. Two 20 µl subsamples were taken from this sample for two C_aCO_2 measurements in a Cameron cell, and 250 µl was used in an AVL Blood Gas Analyzer to measure P_aO_2, P_aCO_2 and pH_a. The remainder was immediately put into an Eppendorf tube, quick-frozen in liquid nitrogen, and then stored at -80°C for [lactate] measurement at the end of the experiment. Experience indicated that PO_2, PCO_2, CCO_2,

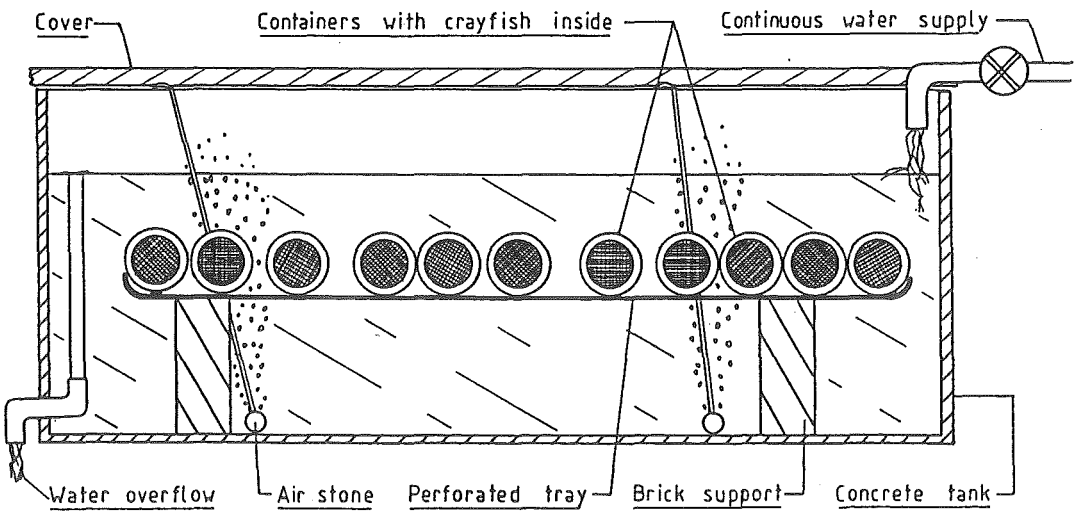


Figure 5.2 A drawing of the experimental tank. The water level is lowered by replacing the upstanding overflow pipe with a pipe short enough to bring the water level just below the supporting tray.

and pH could be measured without clotting if the sample was kept on ice and processed promptly.

The experimental conditions were:-

Settling - which was started between 10.00 am and 11.00 am. Haemolymph samples were taken at the beginning, and after 3 and 8 hours, samples 1, 2, and 3, with the final settling samples taken between 6.00 pm and 7.00 pm.

Emersion - was achieved by lowering the water level, and haemolymph samples were taken at the beginning, and after 3, 12, 24, 36 and 48 hours, samples 4, 5, 6, 7, 8, and 9. Emersion was started as close to 8.00 pm as possible to ensure that samples 4, 6, 7, 8 and 9 were taken as near to 8.00 am and 8.00 pm as possible.

Recovery - was achieved by raising the water level. Recovery of the resubmerged crayfish was monitored, with haemolymph samples taken at the beginning and after 3, 10 and 18 hours, samples 10, 11, 12 and 13.

Controls - a group of animals were settled in water for 48 hours before haemolymph samples were taken, sample 14.

The experimental tank was in the aquarium room, where the animals were usually kept, at 15 ± 1°C with a 12 hour day 12 hour night light cycle. The same animal could not provide the haemolymph samples from settling through to recovery as each sample needed to be a minimum of 0.5 ml and 13 samples were required. Sampling would also upset the crayfish and alter the outcome of subsequent samples. Consequently each crayfish was used only once, ensuring undisturbed and unstressed animals. Up to 40 animals could be accommodated in the experimental tank, and it was possible to do the whole experiment, with 13 sample points, analysing the haemolymph of three crayfish at each sample point. It took 30 to 40 minutes to take and analyze the three haemolymph samples.

The experiment, settling in water, 48 hours aerial respiration and recovery in water, was repeated six times and provided 16 measurements for each of sample points 1 to 12, and 10 for sample point 13. The 29 control animals are a separate group. A total of 231 animals was used.

(3) A.V.L. Blood Gas Analyzer

Measuring PO_2, PCO_2, and pH with three separate electrodes means a large part of a sample is lost in the delivery systems. This makes a machine with the three electrodes connected to a single short delivery system desirable. The advantage is only maintained, however, if all the electrodes are operating reliably, at the same time, and at their proper level of accuracy.

The AVL Blood Gas Analyzer is a piece of medical equipment which can record PO_2, PCO_2 and pH from a sample as small as 200 µl at 37°C and with the range of values found in mammalian blood. The temperature of the AVL electrodes is regulated by water circulating through a heater and thermostat. For the AVL to operate at 15°C a change was required in the circulation to cool the water below ambient, and changes were needed in the electronic thermostat to regulate within a different temperature range. An external water bath was used to cool the circulating water below 15°C (Figure 5.3), and by working this against the thermostated heating block within the AVL it was possible to maintain the temperature constant at 15°C ± 0.1°C provided the temperature within the laboratory did not fluctuate excessively. A Gould recorder was used to monitor temperature fluctuations.

The pH electrode was calibrated with Radiometer precision buffer solutions S1500 and S1510, which read 6.900 and 7.445 respectively at 15°C. The barometric pressure was read twice each day. The PO_2 electrode was checked with a low flow of water saturated air at

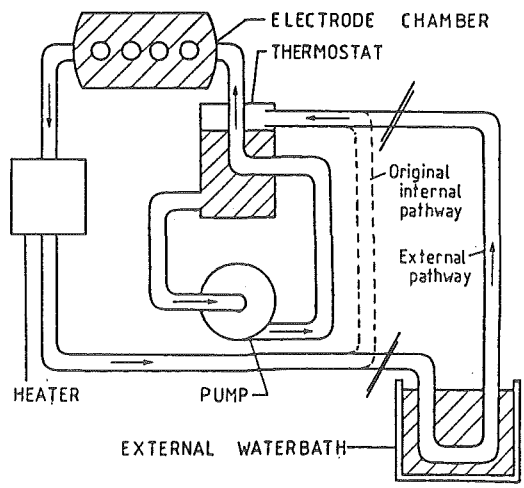


Figure 5.3 A drawing of the change made to the water circulation in the AVL Blood Gas Analyzer to reduce the temperature from 37°C to 15°C.

ambient temperature, which produces condensation when reduced to 15°C. The PCO_2 electrode was calibrated daily with dry air and a cylinder of 5.4% CO_2 in air mixed by a Wösthoff Digamix type 2M 201/a-F gas mixing pump. This mix was humidified and a calibration curve was produced between 0.108% CO_2 (0.807 Torr) and 5.4% CO_2 (40.349 Torr) with AVL scale values between 15 and 200. The experimental haemolymph PCO_2 measurements were read off the curve.

(4) Cameron cell

The CCO_2 of a sample was measured using a 2.8 ml Cameron cell filled with 0.01N HCl, and operating at 40°C (Cameron, 1971). The acidic cell drives the following two reactions to the right, and the total CO_2 is measured as a change in PCO_2.

$$H^+ + CO_3^{-2} \leftrightharpoons HCO_3^-$$

$$H^+ + HCO_3^- \leftrightharpoons H_2CO_3 \leftrightharpoons CO_{2(gas)} + H_2O$$

The change in PCO_2 is measured as a change in pH by a Radiometer PHM 84 Research pH Meter. The cell was calibrated with a 10 $mmol.l^{-1}$ $NaHCO_3$ standard which was prepared daily. Two haemolymph samples of 20 µl were measured between two 20 µl standards. Up to three crayfish (= six standards and six samples) could be processed before the acid in the Cameron cell needed to be replaced.

(5) Y.S.I. L-lactate analyzer

The Lactate concentration was measured using a YSI 23L Lactate analyzer. L-lactate oxidase and flavin adenine dinucleotide (FAD) are used to convert L-lactate and oxygen to hydrogen peroxide and pyruvate, and the hydrogen peroxide in contact with electrodes yields hydrogen ions, oxygen and electrons which provide a current proportional to the concentration of L-lactate in the sample. The reactions are;

$$\text{L-lactate} + O_2 \xrightarrow[\text{L-lactate oxidase}]{\text{FAD}} H_2O_2 + \text{Pyruvate}$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^-$$

The YSI 23L Lactate Analyzer produced the same results as the Boehringer lactate kit (#139 084) when measuring the haemolymph [lactate] in the crab *Ovalipes catharus* (Davidson, *pers. comm.*).

Crayfish haemolymph samples were stored at -80°C in Eppendorf tubes. The samples were thawed on ice and the clot which formed was broken up and the sample centrifuged for 5 minutes in an Eppendorf model 3200 centrifuge. A 25 µl sample of the supernatant haemolymph was taken up with a YSI syringe pipette and injected into the chamber of the lactate analyzer, and the lactate concentration between 0.1 and 15.0 $mmol.l^{-1}$ was read from the display. Any concentration above 15.0 $mmol.l^{-1}$ was diluted with distilled water and measured again. A 5.0 $mmol.l^{-1}$ calibration standard was sampled after six to eight haemolymph samples and a 10.0 $mmol.l^{-1}$ or a 15.0 $mmol.l^{-1}$ standard was run after all values > 10 $mmol.l^{-1}$ to check accuracy.

(6) Data analysis and statistical methods

The haemolymph P_aO_2, P_aCO_2, pH_a, C_aCO_2 and [lactate] of the crayfish at each sampling time were analyzed with oneway Anova and differences between the samples were identified with the Tukey-Kramer Multiple Comparisons Test. Like the other variables, the statistics for the pH are calculated directly, and not as $[H^+]$ (Boutilier and Shelton, 1980).

The daytime and nighttime haemolymph pH of crayfish out of the water was analyzed from morning and evening (8 am and 8 pm) samples with an unpaired two tailed "t" test, comparing morning samples 6 and 8 with evening samples 4,7 and 9, and comparing samples 6 with 7 and 8 with 9.

The data was analyzed with the InStat version 2.04 computer package from GraphPad Software. Results are tested at the 5% level of significance and the data are presented in tables as the mean ± 1 standard deviation, and ± 1 standard error, and graphically as the mean ± 1 standard error (SEM).

III RESULTS

Changes in the haemolymph P_aO_2 observed during the experiment, samples 1 to 13, and the control, sample 14, are summarised in Table 5.1 and plotted in Figure 5.4. The Anova produced a significant result, $F_{(13,210)} = 11.154$, $P < 0.0001$. The control crayfish which had been settled in water for 48 hours had a haemolymph P_aO_2 of 60 Torr. The P_aO_2 of 110 Torr in recently disturbed crayfish, sample 1, and of 100 Torr in the crayfish during the first 10 hours recovering from aerial respiration, samples 10, 11, & 12, differed significantly from the control value. While the crayfish were breathing air for 48 hours the haemolymph P_aO_2 varied between 60 and 30 Torr which was not significantly different from the control value from crayfish settled in water for 48 hours.

During the period of settling there is a non-significant drop in P_aO_2 from 107.8 Torr to 90.0 Torr. At emersion and the beginning of aerial respiration there is a significant drop in P_aO_2 from 90.0 Torr to 55.2 Torr, samples 3 and 4. The lowest P_aO_2 of 32.1 Torr, recorded after 12 hours in air, was significantly different from the settling samples 1, 2 and 3, and the recovery samples 10, 11 and 12, but not the control value, sample 14. When the crayfish return to the water there is a significant increase in P_aO_2 from 44.1 to 98.0 Torr, samples 9 and 10.

Unlike the $\dot{M}O_2$, reported in Chapter 4, which declined quickly during the recovery phase of the experiment, the P_aO_2 remained high for the first 10 hours of the recovery phase before reducing to a level which was not significantly different from the control value.

Changes in the haemolymph P_aCO_2 observed during the experiment, samples 1 to 13, and the control, sample 14, are summarised in Table 5.2 and plotted in Figure 5.5. The Anova produced a significant result, $F_{(13,216)} = 13.693$, $P < 0.0001$. The haemolymph P_aCO_2 of crayfish settling in water, samples 1, 2 and 3, the first aerial sample, sample 4, and recovery samples 10, 11, 12 and 13, were not significantly different from the control group, sample 14, and ranged from 2.6 Torr to 3.6 Torr.

Haemolymph P_aCO_2 increased upon emersion and was significantly different from the control value after three hours aerial respiration, sample 5. The highest mean P_aCO_2 of 6.06 Torr was observed after 12 hours aerial respiration, sample 6, and was significantly higher than all settling, recovery and control values. When the crayfish changed from aerial respiration, sample 9, back to aquatic respiration, sample 10, there was an immediate and significant drop in the haemolymph P_aCO_2. The P_aCO_2 continued to decline rapidly during recovery, and although subsequent changes are not significant relative to the control value, after three hours recovering in water, sample 11, the P_aCO_2 was similar to the control value. The high value of P_aCO_2 recorded after 12 hours in air, sample 6, corresponds to the time of the lowest recorded P_aO_2 value in Table 5.1.

The changes in haemolymph pH_a, summarised in Table 5.3, are plotted in Figure 5.6. The Anova produced a significant result, $F_{(13,199)} = 3.335$, $P = 0.0001$. The animals in the control group recorded a mean pH_a of 7.64 and there are no experimental samples which are significantly different from this. The haemolymph pH_a of 7.55 after 12 hours aerial respiration, sample 6, and pH_a of 7.58 after 36 hours aerial respiration, sample 8, were the two lowest recorded. Both were early morning samples. Sample 6 with a pH_a of 7.55 was significantly different from settling sample 2, with a pH_a of 7.69, and recovery samples 11, 12 and 13, with pH_a values of 7.71, 7.68 and 7.74 respectively. The highest recorded pH_a of 7.74 is sample 13, the final recovery sample, but it is not significantly different from the control value.

The lowest pH_a of 7.55 recorded after 12 hours in air, sample 6, is at the same time as the lowest haemolymph P_aO_2 and the highest P_aCO_2 recorded.

The difference between the haemolymph pH in the morning, samples 6 and 8, and the pH in the evening, samples 4, 7 and 9, was extremely significant ($t = 3.73$, 73 df, $P = 0.0004$). There were very significant differences between samples 6 and 7 ($t = 2.888$, 27 df, $P = 0.0076$), and between samples 8 and 9 ($t = 2.997$, 28 df, $P = 0.0057$). These results indicate that haemolymph pH in the evening is significantly more

alkaline than in the morning, by 0.1 pH units, Table 5.3.

The total carbon-dioxide content of the haemolymph, C_aCO_2, is the sum of the dissolved CO_2 gas, HCO_3^- and CO_3^{-2}. The haemolymph C_aCO_2, and the effect of aerial exposure is summarised in Table 5.4 and plotted in Figure 5.7. The Anova produced a significant result, $F_{(13,214)} = 14.253$, $P < 0.0001$. The control crayfish kept settled in water for 48 hours had a haemolymph C_aCO_2 of 12.46 $mmol.l^{-1}$. The haemolymph C_aCO_2 was significantly different from the control group for crayfish in air for 12, 24, 36 and 48 hours, samples 6, 7, 8, and 9, and when first resubmerged in water, sample 10. Haemolymph C_aCO_2 was not significantly different from the control group for the crayfish settling in water, samples 1, 2 and 3, the first three hours aerial respiration, samples 4 and 5, and all but the first of the samples from recovery in water, samples 11, 12 and 13.

When crayfish start breathing air they show an immediate increase in C_aCO_2, and after 24 hours it is 17.68 $mmol.l^{-1}$, more than 40 % above the control value of 12.46 $mmol.l^{-1}$. After 36 hours and 48 hours in air haemolymph C_aCO_2 was 35 % and 47 % above the control value respectively. There was an extremely rapid drop in haemolymph C_aCO_2 when the crayfish were returned to the water, and within 3 hours the C_aCO_2 was not significantly different from the control value.

Changes in haemolymph lactate concentrations recorded during the experiment are presented in Table 5.5 and Figure 5.8. The Anova produced a significant result, $F_{(13,209)} = 14.647$, $P < 0.0001$. Control crayfish kept settled in water for 48 hours had a haemolymph [lactate] of 0.12 $mmol.l^{-1}$. All the samples, except sample 6, have [lactate] which is not significantly different from the control value. There was a 40 fold increase in [lactate] above the control value after 12 hours aerial respiration, sample 6. In the next 12 hours, however, the [lactate] declined and remained at 2 to 3 times the control value for the latter half of the 48 hours aerial respiration.

The high haemolymph [lactate] after 12 hours in air is at the same time as the highest P_aCO_2 and the lowest P_aO_2 and pH_a recorded.

Table 5.1 Summary of the changes in crayfish haemolymph P_aO_2 during settling in water, 48 hours in air, and recovery in water. The control animals were settled in water for 48 hours. The column "p < 0.05" lists the significant pairwise comparisons between the samples.

	-- sample --			P_aO_2	± stdev	± S E M	p < 0.05
	no	hour	n	----- torr -----			
Settling	1	0	15	107.8	31.21	8.06	4,5,6,7,8,9,14
in	2	3	16	80.0	45.45	11.36	6,9
water	3	8	16	90.0	27.22	6.80	4,6,7,9
	4	10	16	55.2	34.75	8.69	1,3,10,11,12
Breathing	5	13	15	61.1	29.32	7.57	1,10,11,12
air	6	22	15	32.1	25.71	6.64	1,2,3,10,11,12
for	7	34	15	48.0	30.81	7.95	1,3,10,11,12
48 hours	8	46	15	58.0	23.43	6.05	1,10,11,12
	9	58	15	44.1	28.75	7.42	1,2,3,10,11,12
Recovery	10	60	16	98.0	23.75	5.94	4,5,6,7,8,9,14
in	11	63	16	101.6	24.16	6.04	4,5,6,7,8,9,14
water	12	70	15	101.5	22.96	5.93	4,5,6,7,8,9,14
	13	78	10	70.5	18.89	5.97	NS
Control	14		29	62.2	25.15	4.67	1,10,11,12

Oneway Anova $F_{(13,210)} = 11.154$, $P < 0.0001$

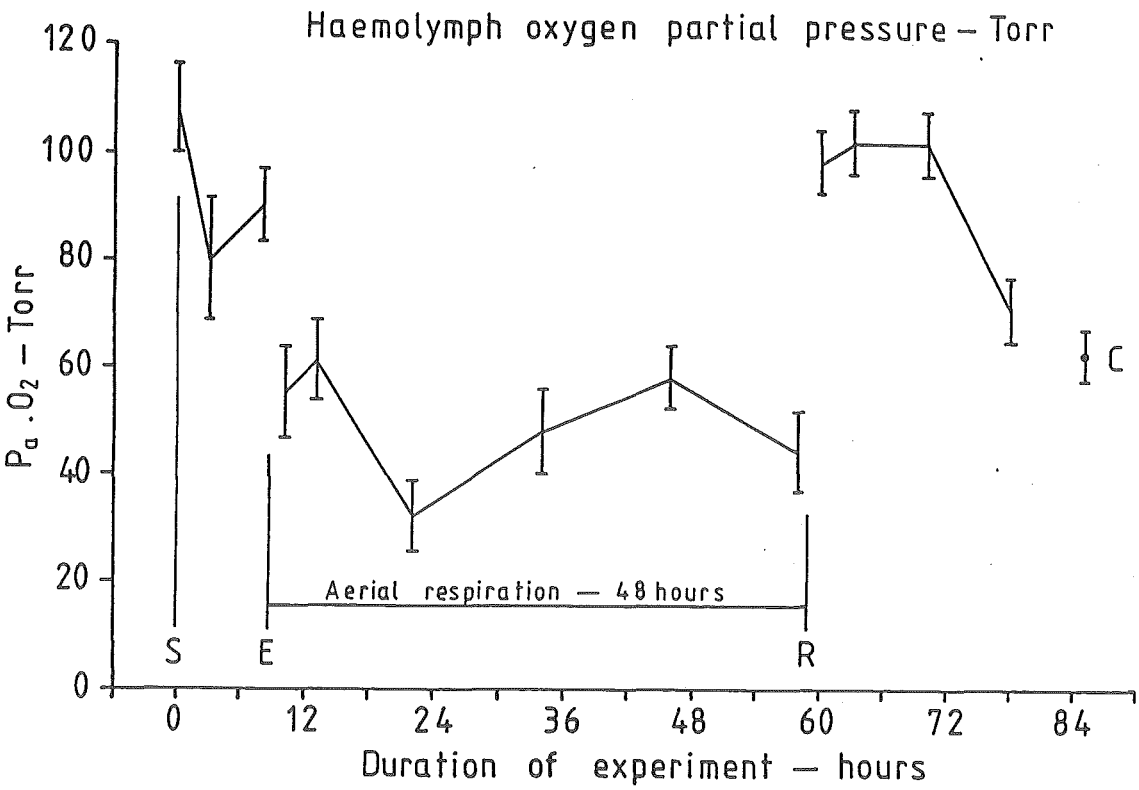


Figure 5.4 Plot of haemolymph P_aO_2 from Table 5.1. **S** - start settling in the water; **E** - is emersion and aerial respiration; **R** - start recovery; **C** - control value. Data are mean ± SEM.

Table 5.2 Summary of the changes in crayfish haemolymph P_aCO_2 during settling in water, 48 hours in air, and recovery in water. The control animals were settled in water for 48 hours. The column "p < 0.05" lists the significant pairwise comparisons between the samples.

	-- sample --			P_aCO_2	± stdev	± S E M	p < 0.05
	no	hour	n	----- torr -----			
Settling	1	0	16	2.67	0.79	0.20	6,7,9
in	2	3	16	3.60	1.61	0.40	6,7
water	3	8	16	2.63	1.25	0.31	6,7,9
	4	10	16	2.97	1.25	0.31	6,7,9
Breathing	5	13	16	4.11	1.78	0.44	6,11,12,14
air	6	22	16	6.06	1.96	0.49	1,2,3,4,5,8,10,11,12,13,14
for	7	34	16	5.29	2.33	0.58	1,2,3,4,10,11,12,13,14
48 hours	8	46	16	4.18	1.28	0.32	6,11,12,14
	9	58	16	5.07	1.81	0.45	1,3,4,10,11,12,13,14
Recovery	10	60	16	3.36	0.78	0.19	6,7,9
in	11	63	16	2.34	0.78	0.19	5,6,7,8,9
water	12	70	15	2.08	0.74	0.19	5,6,7,8,9
	13	78	10	2.57	0.42	0.13	6,7,9
Control	14		29	2.59	0.53	0.10	5,6,7,8,9

Oneway Anova $F_{(13,216)} = 13.693$, $P < 0.0001$

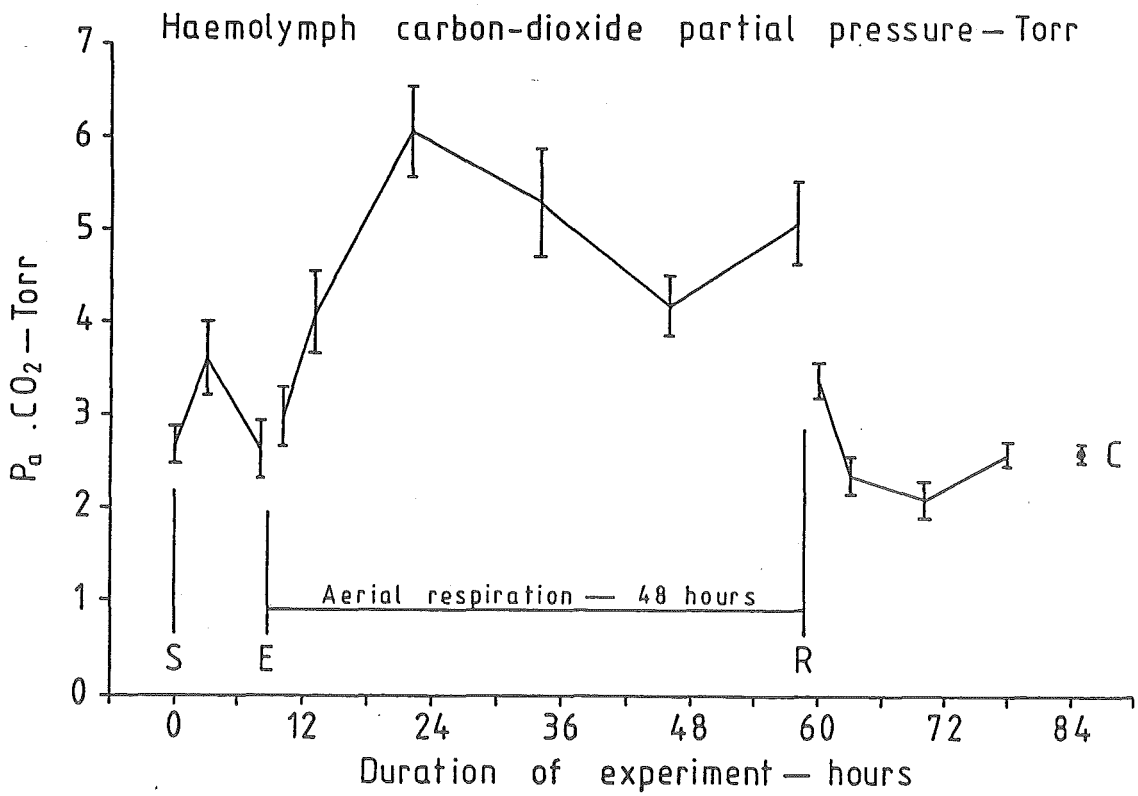


Figure 5.5 Plot of haemolymph P_aCO_2 from Table 5.2. **S** - start settling in the water; **E** - is emersion and aerial respiration; **R** - start recovery; **C** - control value. Data are mean ± SEM.

Table 5.3 Summary of the changes in crayfish haemolymph pH_a during settling in water, 48 hours in air, and recovery in water. The control animals were settled in water for 48 hours. The column "p < 0.05" lists the significant pairwise comparisons between the samples.

	-- sample -- no	hour	n	pH_a	± stdev	± S E M	p < 0.05
Settling	1	0	16	7.63	0.093	0.023	NS
in	2	3	10	7.69	0.095	0.030	6
water	3	8	14	7.62	0.072	0.019	NS
	4	10	16	7.62	0.088	0.022	NS
Breathing	5	13	15	7.65	0.103	0.027	NS
air	6	22	14	7.55	0.086	0.023	2,11,12,13
for	7	34	15	7.65	0.089	0.023	NS
48 hours	8	46	16	7.58	0.098	0.025	11,13
	9	58	14	7.68	0.073	0.020	NS
Recovery	10	60	13	7.65	0.098	0.027	NS
in	11	63	16	7.71	0.116	0.029	6,8
water	12	70	15	7.68	0.118	0.031	6
	13	78	10	7.74	0.060	0.019	6,8
Control	14		29	7.64	0.121	0.022	NS

Oneway Anova $F_{(13,199)} = 3.335$, $P = 0.0001$

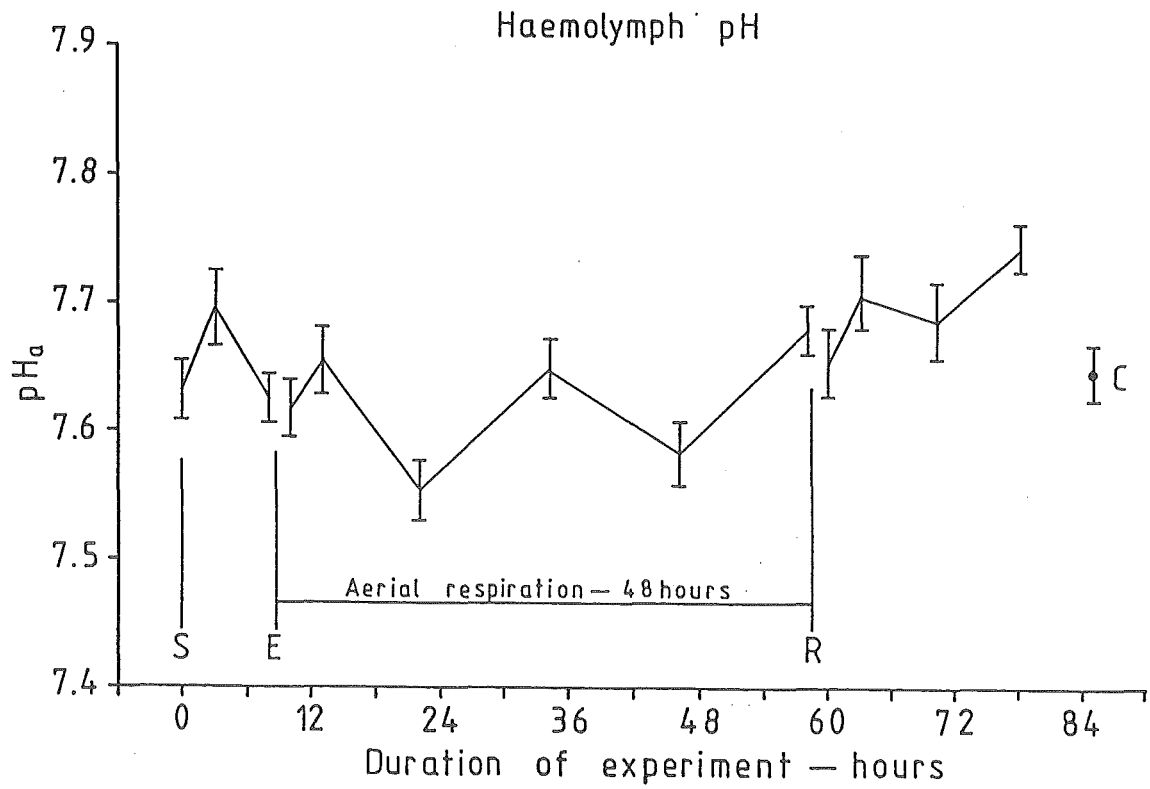


Figure 5.6 Plot of haemolymph pH_a from Table 5.3. **S** - start settling in the water; **E** - is emersion and aerial respiration; **R** - start recovery; **C** - control value. Data are mean ± SEM.

Table 5.4 Summary of the changes in crayfish haemolymph C_aCO_2 during settling in water, 48 hours in air, and recovery in water. The control animals were settled in water for 48 hours. The column "p < 0.05" lists the significant pairwise comparisons between the samples.

	-- Sample --			C_aCO_2	± stdev	± S E M	p < 0.05
	no	hour	n	---- m mol/l ----			
Settling	1	0	16	10.71	2.390	0.597	6,7,8,9,10
in	2	3	16	11.35	2.106	0.527	6,7,8,9,10
water	3	8	16	10.61	2.313	0.578	6,7,8,9,10
	4	10	16	11.55	2.228	0.557	6,7,8,9,10
Breathing	5	13	15	13.81	2.640	0.682	7,9
air	6	22	16	15.83	2.228	0.557	1,2,3,4,12,14
for	7	34	16	17.68	4.822	1.206	1,2,3,4,5,11,12,13,14
48 hours	8	46	16	16.85	3.484	0.871	1,2,3,4,12,13,14
	9	58	15	18.28	3.173	0.819	1,2,3,4,5,11,12,13,14
Recovery	10	60	16	16.62	2.362	0.591	1,2,3,4,12,13,14
in	11	63	16	13.60	3.361	0.840	7,9
water	12	70	15	11.88	2.924	0.755	6,7,8,9,10
	13	78	10	12.18	1.928	0.610	7,8,9,10
Control	14		29	12.46	2.593	0.482	6,7,8,9,10

Oneway Anova $F_{(13,214)} = 14.253$, $P < 0.0001$

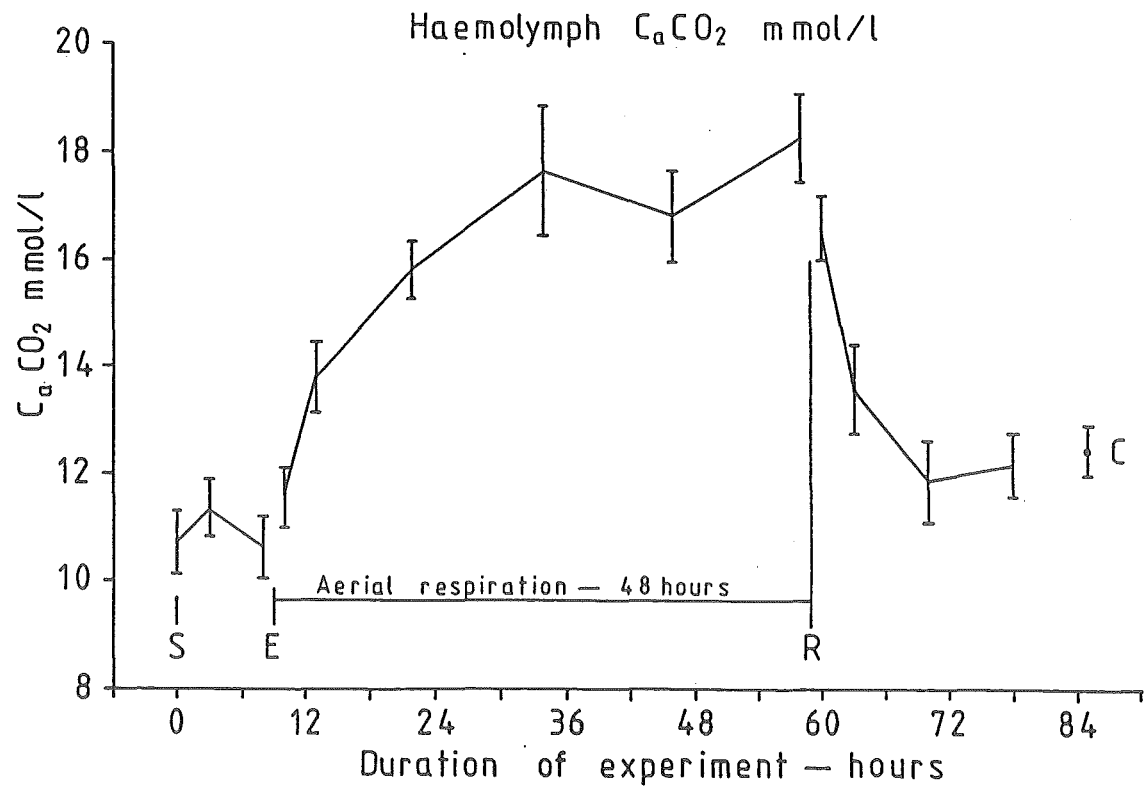


Figure 5.7 Plot of haemolymph C_aCO_2 from Table 5.4. **S** - start settling in the water; **E** - is emersion and aerial respiration; **R** - start recovery; **C** - control value. Data are mean ± S E M.

Table 5.5 Summary of the changes in crayfish haemolymph [lactate] during settling in water, 48 hours in air, and recovery in water. The control animals were settled in water for 48 hours. The column "p < 0.05" lists the significant pairwise comparisons between the samples.

	-- sample --			Lactate	± stdev	± S E M	p < 0.05
	no	hour	n	----- mmol/l -----			
Settling	1	0	16	0.45	0.38	0.10	6
in	2	3	16	0.36	0.38	0.09	6
water	3	8	15	0.11	0.09	0.02	6
	4	10	16	0.15	0.08	0.02	6
Breathing	5	13	16	1.24	2.21	0.55	6
air	6	22	16	4.85	4.07	1.02	1 to 14
for	7	34	15	0.29	0.32	0.08	6
48 hours	8	46	14	0.29	0.27	0.07	6
	9	58	14	0.37	0.43	0.12	6
Recovery	10	60	15	0.33	0.58	0.15	6
in	11	63	16	0.43	0.25	0.06	6
water	12	70	15	0.74	0.87	0.22	6
	13	78	10	0.16	0.12	0.04	6
Control	14		29	0.12	0.10	0.02	6

Oneway Anova $F_{(13,209)} = 14.647$, $P < 0.0001$

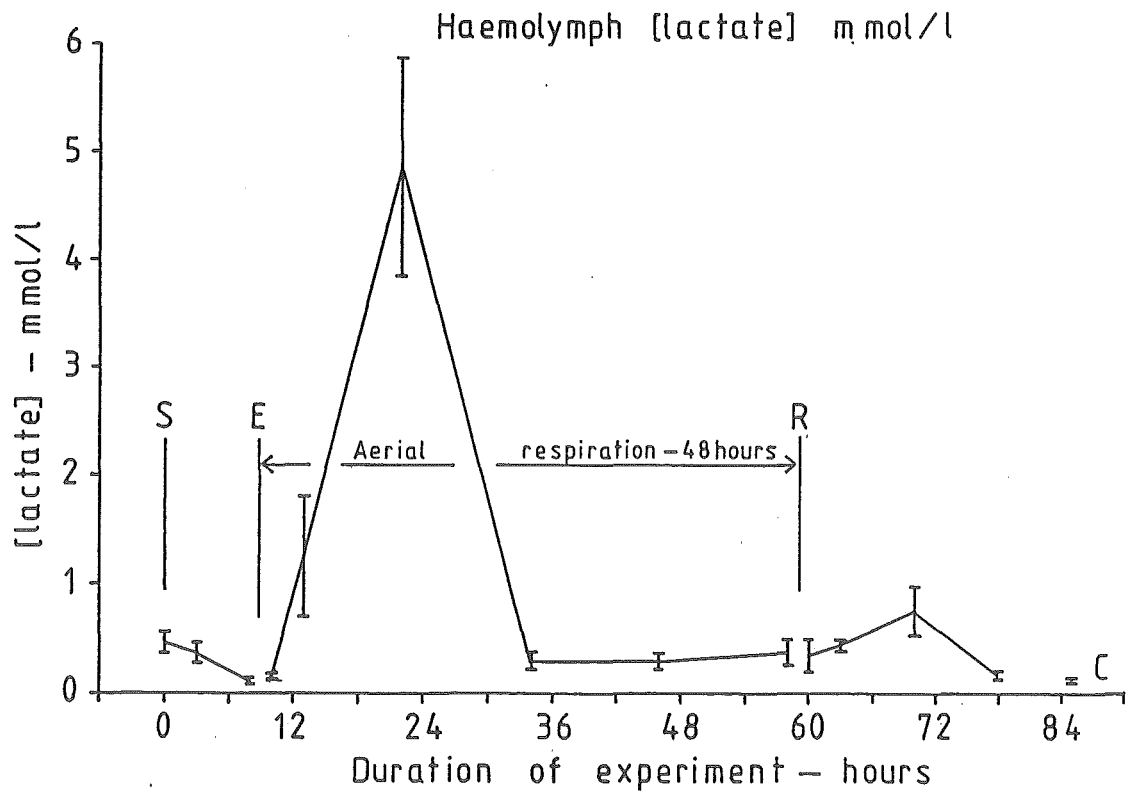


Figure 5.8 Plot of haemolymph [lactate] from Table 5.5. **S** - start settling in the water; **E** - is emersion and aerial respiration; **R** - start recovery; **C** - control value. Data are mean ± SEM.

IV DISCUSSION

It was demonstrated in Chapter 4 that crayfish were settled after eight hours in water as the $\dot{M}O_2$ of 1.1 $\mu mol.g^{-1}.h^{-1}$ was similar to the $\dot{M}O_2$ of 1.07 $\mu mol.g^{-1}.h^{-1}$ at P_{CRIT}, Chapter 6, when the rate of oxygen uptake can no longer be maintained by crayfish in the face of increasing hypoxia. After eight hours settling in water the haemolymph P_aCO_2, pH_a, and C_aCO_2 had returned to initial values, Tables 5.2 to 5.4 and Figures 7.5 and 7.7, and the haemolymph P_aO_2, P_aCO_2, pH_a, C_aCO_2 and [lactate] were not significantly different from control values measured from crayfish settled in water for 48 hours, Tables 5.1 to 5.5, indicating that the crayfish are settled.

The initial high P_aO_2 of 108 Torr, compared with the control value of 62 Torr, suggests a period of elevated ventilation initiated by the stress of being handled in air, dried, weighed, put into the respirometer and returned to the water. The small drop in P_aO_2 and small rise in P_aCO_2 and C_aCO_2, after three hours settling suggest a temporary reduction in ventilation during the 8 hours settling period.

When the crayfish left the water and started breathing air there was an initial significant drop in P_aO_2 from 90 Torr to 55 Torr. This drop in haemolymph P_aO_2 indicates that even though the environment now contains 21% oxygen there has been a change to oxygen transfer at the respiratory surface. The results in Chapter 4 indicate that there is no change in $\dot{M}O_2$ when crayfish emerge; therefore the decrease in P_aO_2 must be caused by poor ventilation or increased resistance to diffusion. Poor ventilation of the branchial chamber, besides depleting branchial chamber oxygen content, resulting in a low haemolymph P_aO_2, would also cause an elevation of the PCO_2 in the branchial chamber, which was not indicated by the haemolymph P_aCO_2 between 4 and 6 Torr.

When a crayfish emerges from the water a large part of the water contained within the branchial chamber drains out, leaving the gills covered by a film of water and clumped together by water surface tension (Taylor and Wheatly, 1980).

It was suggested in Chapter 4 that this could increase the resistance to diffusion and in order to maintain $\dot{M}O_2$ the diffusion gradient would need to be increased. The reduction in P_aO_2 from 90 Torr to 55 Torr when crayfish emerged from the water increased the air to haemolymph diffusion gradient by 35 Torr. If the PO_2 of the inspired air is between 155 and 160 Torr then the 35 Torr increase represents a 50% increase, approximately, in the diffusion gradient. The immediate increase in haemolymph P_aO_2, to 100 Torr, when the crayfish were returned to the water indicates that it was not O_2 availability, which is low in water, but resistance to the transfer of O_2 to the haemolymph which causes the drop in haemolymph P_aO_2.

During the first three hours of aerial respiration there were small non significant increases in the haemolymph P_aO_2, P_aCO_2, pH_a, C_aCO_2 and [lactate], samples 4 to 5. The crucial period was between 3 and 12 hours in air, when the P_aO_2 just above 30 Torr and pH_a of 7.55 were the lowest recorded, and the P_aCO_2 of 6 Torr was the highest recorded. There was an accumulation of lactate during the first 12 hours in air to a concentration of 5 $mmol.l^{-1}$, and the C_aCO_2, at 16 $mmol.l^{-1}$, was still increasing. The expected respiratory acidosis, an increase in P_aCO_2, C_aCO_2 and $[H^+]$, occurred during the first 12 hours in air. The [lactate] declined in the subsequent 12 hours and remained at levels similar to the control value. After 12 hours in air the haemolymph P_aO_2 settled between 40 and 60 Torr and the P_aCO_2 settled between 4 and 5.3 Torr.

Changes in the P_aCO_2, C_aCO_2 the $[H^+]$ are summarised in the Davenport diagrams Figures 7.4 to 7.7, and are a series of shifts between metabolic alkalosis and metabolic acidosis. The oscillation, of up to 0.1 pH units, while the crayfish is in air appears to be more than acid-base changes in response to P_aCO_2.

Considerable work has been done on the circadian and diel rhythms of Crustacea and Quilter (1975) found that *P. zealandicus* had a diel activity rhythm, and was most active in the middle of the night. Activity measurements in Chapter

3 indicate that solitary crayfish and crayfish pairs, at 18°C, show higher levels of emersion activity at night than during the daytime.

Massabuau *et al.* (1984) found that the ventilatory requirement of *Astacus leptodactylus* was higher in the evening than in the morning. Sakakibara *et al.* (1987) argue that this was to adjust the haemolymph pH by ventilatory control of haemolymph PCO_2, and found that the haemolymph pH of *A. leptodactylus* was 0.1 units more alkaline at dusk than at dawn. It was suggested that the alkalosis was linked to an increase in metabolism, inducing a Bohr effect when oxygen demand was increasing (Sakakibara et al., 1987).

When *P. zealandicus* was in the air the haemolymph pH_a appeared to change in a regular manner, independent of the [lactate] and an elevated P_aCO_2 and C_aCO_2, and the Davenport diagrams, Figures 7.4 and 7.6, indicate that there was a large metabolic component in the pH regulation. The ability of the crayfish to maintain pH_a regulation while they were out of the water indicates a regulation of haemolymph pH independent of P_aCO_2, C_aCO_2 and the constraints of aerial respiration.

Studies of the changes in the haemolymph of aquatic crustacea during periods of exposure to air note changes in P_aO_2, and declining $\dot{M}O_2$ with an associated increase in [lactate] during the initial stages of aerial exposure while water is draining from the branchial cavities and the gills are drying and separating (Taylor and Wheatly, 1981; Morris *et al.*, 1986c; Vermeer, 1987; Taylor and Whiteley, 1989). Lactate levels reached 8 $mmol.l^{-1}$ in *A. pallipes* (Taylor and Wheatly, 1981; Morris *et al.*, 1986b), 6 $mmol.l^{-1}$ in *Homarus gammarus* (Taylor and Whiteley, 1989), and 5.5 $mmol.l^{-1}$ in *Panulirus argus* (Vermeer, 1987). Taylor and Wheatly (1981) found that the haemolymph oxygen content of *A. pallipes* fell to almost zero when first in air and they consider that this was caused by water in the branchial chambers taking up to one hour to drain away, preventing aerial gas exchange. This internal hypoxia resulted in a considerable increase in [lactate] from 0.5 to 8 $mmol.l^{-1}$. In *P. zealandicus*, the lack

of such a dramatic increase in [lactate] or fall in $\dot{M}O_2$, and the small rise in P_aCO_2 and C_aCO_2 in the first few hours, all indicate that the water drains quickly from the branchial chamber permitting aerial respiration to start. An initial drop in P_aO_2 down to 55 Torr was experienced by *P. zealandicus* which is much higher than the P_aO_2 of 10 Torr recorded from *A. pallipes* (Taylor and Wheatly, 1981). The most stressful time recorded for *P. zealandicus* appeared to be about 12 hours after leaving the water when [lactate] reached 5 $mmol.l^{-1}$, which is 40 times the settled aquatic [lactate].

As crayfish settled in air have an $\dot{M}O_2$ which is not significantly different from the $\dot{M}O_2$ settled in water, there would need to be a non-discernable increase in activity, eg. an increase in heart or scaphognathite rate, to account for an appearance of lactate. The accumulation of the lactate indicated that oxygen transport was not meeting the metabolic needs at some time during the first 12 hours in air. After 12 hours in air the $\dot{M}O_2$ was still at the settled level, and if resistance to oxygen diffusion at the gills was limiting $\dot{M}O_2$ then the lowest recorded P_aO_2 of 32 Torr suggests that resistance at this time must be at its highest. It is possible that greater respiratory stresses were experienced earlier, between 3 hours in air and 12 hours in air, and the crayfish were already recovering when the samples were taken.

It can only be assumed that during the first 12 hours in air there was a period when energy needs exceeded the aerobic capacity. As the crayfish have been shown to be night active in Chapter 3, and the sample after 12 hours in air was taken at 8 am, the accumulated lactate may be the consequence of elevated nocturnal metabolic activity coming on top of the initial changes associated with emersion. That there was an adequate oxygen supply to re-metabolise the lactate at a later time is indicated by the removal of the lactate from the haemolymph in the next 12 hours. There was no "surge" in [lactate] upon re-immersion suggesting that the lactate had not been retained in the tissues.

There were some rapid changes in the haemolymph when the crayfish were returned to the water and resumed aquatic

respiration. The P_aO_2 increased from 45 to 100 Torr and the P_aCO_2 decreased from 5 to 3.4 Torr, and within 3 hours was at 2.3 Torr. There were small drops in pH_a and C_aCO_2, and no change in [lactate]. The P_aO_2 remained elevated at 100 Torr for at least 10 hours during recovery in water, much longer than during the settling period. The P_aCO_2 and C_aCO_2 decreased very quickly, falling below the control value within 10 hours, but both P_aCO_2 and C_aCO_2 were similar to the control value after 18 hours in the water. The pH_a of 7.74, after 18 hours recovery, was the highest recorded, and the [lactate] was similar to the control value.

In Chapter 4 it was calculated that the oxygen consumed during recovery from aerial respiration was similar to that required during the settling period. The elevated haemolymph P_aO_2 of 100 Torr during the first 10 hours recovering in water did not appear to serve respiration as the rapidly declining $\dot{M}O_2$ had not depleted the haemolymph oxygen content. When the crayfish were returned to the water the extremely rapid decline in P_aCO_2 suggested that haemolymph CO_2 was being removed by respiratory means. An elevated respiration would keep the P_aO_2 elevated. The removal of CO_2 at the gills appears to have been maintained until the haemolymph C_aCO_2 had returned to normal, a decline from 18 to 12 $mmol.l^{-1}$, after which there was an increase in P_aCO_2 and a decline in P_aO_2 to control values, between 10 and 18 hours in water.

The crayfish *A. pallipes* after three hours emersion (Taylor and Wheatly, 1980), and after 24 hours emersion (Taylor and Wheatly, 1981), experienced increases in f_R, f_H, and $\dot{M}O_2$ which resembled the response of crayfish settling in water after being disturbed at the beginning of the experiment. During the period of recovering in water, after 24 hours in air, the P_aO_2 was elevated above settled submerged levels; P_aCO_2 returned to settled levels almost immediately, and there was a very rapid decline in C_aCO_2 (Taylor and Wheatly, 1981).

In *P. zealandicus* the pattern of $\dot{M}O_2$ changes during settling and during the recovery from aerial respiration were similar, with no evidence of an accumulated oxygen debt

other than the reduced haemolymph oxygen content. The changes in P_aO_2 while settling and recovering from aerial respiration were different. The difference stems from the haemolymph C_aCO_2 of 12 mmol.l^{-1} at the beginning of settling and the C_aCO_2 of 18 mmol.l^{-1} at the beginning of recovery.

The haemolymph changes suggest that after 48 hours in air *P. zealandicus* required a recovery period of at least 10 hours, and the nature of the changes in P_aCO_2, pH_a and C_aCO_2 indicate that this was not to recover an oxygen debt but to remove the excess carbonates. The experiments on voluntary emersion in Chapter 3 indicate that *P. zealandicus* regularly left the water at night, and rapid removal of carbonates from the haemolymph before the next night will be necessary if an accumulation of the carbonate load is to be prevented.

P. zealandicus settled in water had a haemolymph P_aO_2 of 60 Torr, which was similar to the haemolymph P_aO_2 of *Carcinus maenas* (75 Torr) and *Homarus gammarus* (50 Torr) both aquatic Crustacea (Table 5.6). The haemolymph P_aO_2 of *P. zealandicus* in air was between 30 and 60 Torr, which compares with the haemolymph P_aO_2 of *Holthuisana transversa* (56 Torr) and *Birgus latro* (44 Torr), both very terrestrial species with lungs and gills (Greenaway and Taylor, 1976; Greenaway *et al.*, 1988). The aquatic species *A. pallipes* and *H. gammarus* both had low haemolymph P_aO_2 of 10 Torr when in air, and *C. maenas* had a haemolymph P_aO_2 < 20 Torr. This would suggest that while *P. zealandicus* is aquatic, its haemolymph P_aO_2 while in air puts it in the same class as some well developed air breathers.

The change in haemolymph P_aCO_2 after *P. zealandicus* had been in air for 12 hours reached the maximum value of 6 Torr, an increase from 2.5 Torr. This is similar to the change seen in *H. transversa* and *Cyclograpsus lavauxi*, and smaller than the change seen in *H. gammarus*, 3.3 to 9 Torr, or *A. pallipes*, 3 to 10.6 Torr. The large increases in P_aCO_2 experienced by *H. gammarus* and *A. pallipes*, as well as the low values for the P_aO_2, suggest some degree of impaired gas exchange, either at the respiratory surface, or exchange with the environment outside the animal.

Table 5.6 Haemolymph changes in Crustacea exposed to air for varying lengths of time. All values are the mean ± 1 S.E. An * indicates a calculated value.

	P_aO_2 Torr	P_aCO_2 Torr	pH_a	[lactate] mmol/l	C_aCO_2 mmol/l	$[HCO_3^- + CO_3^{-2}]$ mEq/l	Source
Paranephrops zealandicus							
Settled in water	62.2 ± 4.67	2.59 ± 0.1	7.63 ± 0.012	0.12 ± 0.10	12.46 ± 0.48		This study
12 hours in air	32.1 ± 6.64	6.06 ± 0.49	7.54 ± 0.015	4.85 ± 1.02	15.83 ± 0.56		
24 hours in air	48.0 ± 7.95	5.29 ± 0.58	7.64 ± 0.013	0.29 ± 0.08	17.68 ± 1.21		
48 hours in air	44.1 ± 7.42	5.07 ± 0.45	7.67 ± 0.009	0.37 ± 0.12	18.28 ± 0.82		
Austropotamobius pallipes							
Settled in water	33.0 ± 5.0	3.03 ± 0.24	7.896 ± 0.024	0.32 ± 0.06	7.10 ± 0.40		Taylor and Wheatly (1980)
3 hours in air	11.0 ± 1.0	8.89 ± 0.69	7.452 ± 0.024	2.93 ± 0.31	7.96 ± 0.69		
Settled in water	38.4 ± 3.2	3.03 ± 0.24	7.896 ± 0.024	0.55 ± 0.15		7.49 ± 0.54	Taylor and Wheatly (1981)
12 hours in air		10.62 ± 1.08	7.457 ± 0.038	8.28 ± 0.86		12.68 ± 0.42	
24 hours in air	10.0 ± 0.5	7.84 ± 0.82	7.786 ± 0.016	0.57 ± 0.16			
Homarus gammarus							
Settled in water	48.8 ± 11.3	3.30 ± 0.22	7.78 ± 0.02	0.9 ± 0.1		9.3 ± 0.6	Taylor and Whiteley (1989)
3 hours in air	12.0 ± 2.25	5.25 ± 0.60	7.64 ± 0.05	1.8 ± 0.2		10.7 ± 0.8	
14 hours in air	9.0 ± 0.75	9.00 ± 2.25	7.63 ± 0.05	6.2 ± 1.3		15.8 ± 0.5	

Table 5.6 cont. Haemolymph changes in Crustacea exposed to air for varying lengths of time. All values are the mean ± 1 S.E. An * indicates a calculated value.

	P_aO_2 Torr	P_aCO_2 Torr	pH_a	[lactate] mmol/l	C_aCO_2 mmol/l	$[HCO_3^- + CO_3^{-2}]$ mEq/l	Source
Holthuisana transversa							
Settled in water	17.8 ± 2.03	6.0 ± 0.51	7.33 ± 0.029		9.79	9.54 ± 0.73	Greenaway, Bonaventura and Taylor (1983)
Settled in air	56.1 ± 8.9	9.6 ± 0.94	7.41 ± 0.028		13.05	12.66 ± 0.66	Greenaway, Taylor and Bonaventura (1983)
Birgus latro							
Settled in air	43.8 ± 28.9	7.10 ± 0.69	7.731 ± 0.047	0.20 ± 0.20	13.6 ± 2.2		Greenaway *et al* (1988)
Settled in air	78.6 ± 11.23	6.2	7.502 ± 0.040		14.1 ± 1.95		Cameron and Mecklenburg (1973)
Cyclograpsus lavauxi							
Settled in water		2.31 ± 0.40 *	7.922 ± 0.023		10.87 ± 0.83		Innes *et al* (1986)
1-2 hours in air		3.84 ± 0.44 *	7.891 ± 0.045		16.07 ± 0.80		
3-6 hours in air		3.93 ± 0.30 *	7.880 ± 0.024		15.56 ± 1.15		
24-26 hours in air		6.42 ± 0.40 *	7.931 ± 0.029		31.37 ± 1.80		
Carcinus maenas							
Settled in water	74.9 ± 7.3	1.9 ± 0.2	7.843 ± 0.008	3.05 ± 0.15			Taylor and Butler (1978)
2-3 hours in air	18.8 ± 2.0	3.6 ± 0.4	7.315 ± 0.002	3.74 ± 0.52			

The high shore crab *C. lavauxi*, like the crayfish *P. zealandicus*, experienced a large increase in C_aCO_2 when it moved from the water to air, with minimal changes to the pH_a. The more aquatic species, like *A. pallipes* and *H. gammarus* experience larger changes in pH_a.

If a crustacean is to be successfully bimodal it would appear to be necessary to have an ability to maintain a high haemolymph P_aO_2 and low P_aCO_2 when using aerial respiration, and have an ability to minimise the potential respiratory acidosis which accompanies emersion, and the alkalosis which accompanies a return to aquatic respiration. The conditions which enable respiration to be maintained without serious compromise to $\dot{M}O_2$, would also mean that the animal is not likely to face large increases in [lactate].

Conclusions

Resting $\dot{M}O_2$ in air was the same as in water, indicating that the move from aquatic to aerial respiration did not impair the ability of the crayfish to obtain oxygen, but the decline in haemolymph P_aO_2, from 90 Torr to 55 Torr, indicated that an increase in the diffusion gradient from the medium to the haemolymph was needed to maintain the $\dot{M}O_2$ in air.

During recovery in water the crayfish maintained an elevated haemolymph P_aO_2 for 10 hours which was associated with a very rapid drop in P_aCO_2 and C_aCO_2. This was different from the pattern seen when the crayfish were settling at the beginning of the experiment, and was attributed to the increased respiratory effort involved in the removal of haemolymph CO_2.

After 12 hours in air the [lactate] of 5 $mmol.l^{-1}$ differed significantly from the settled level of 0.1 $mmol.l^{-1}$. The accumulated lactate was considered to be the consequence of an elevated nocturnal metabolism of the night active crayfish, coinciding with the initial changes associated with emersion, resulting in the energy needs exceeding the aerobic capacity. The lactate was re-metabolised in the next 12 hours indicating that there was sufficient oxygen available at a later time.

The crayfish *P. zealandicus* demonstrated regular pH_a oscillations, in which the dusk pH_a recorded at 8 pm. was 0.1 pH units more alkaline than the dawn pH_a recorded at 8 am. The crayfish were able to maintain this regulation of pH_a, while they were out of the water, independently of the changes in P_aCO_2, P_aCO_2 and [lactate]. These oscillations have been reported in other crayfish, and are associated with increased activity and metabolism. It has been suggested that the pH change induces a Bohr effect when oxygen demand and activity are increasing at dusk.

CHAPTER 6

THE EFFECT OF HYPOXIA: THE CRITICAL OXYGEN TENSION

I INTRODUCTION

The most ancient organisms are considered to have been anaerobic as there was no oxygen in the atmosphere. Although many organisms now use aerobic metabolism they still have a residual capacity to derive some energy anaerobically. In terrestrial and aquatic creatures anaerobic metabolism is used when the energy needs exceed the capacity of the aerobic energy supply as a result of high levels of activity or low oxygen availability. The use of anaerobic metabolism to supplement energy needs is observed in both water and air breathing creatures during periods of intense activity. The use of anaerobic metabolism to supplement basal metabolism when oxygen is in short supply is often observed in water breathers and less frequently in air breathers as air, except at high altitudes, normally has plenty of available oxygen.

Consequently many studies of water breathing organisms involve an attempt to determine a point called the critical oxygen tension, P_{CRIT}, described in Herreid (1980) as that oxygen tension below which the animal's $\dot{M}O_2$ (rate of oxygen consumption) becomes dependent upon P_IO_2 (the partial pressure of oxygen in the inspired water). This definition suggests that down to the point P_{CRIT} an animal is regulating and able to maintain its $\dot{M}O_2$ at a level sufficient to meet its needs. However as Herreid (1980) points out and other authors demonstrate, P_{CRIT} is not a fixed point and does not identify an abrupt transition from regulating $\dot{M}O_2$ to the condition where $\dot{M}O_2$ depends on P_IO_2 (Spoek, 1974; Taylor *et al.*, 1973, 1977a; Taylor, 1976; Taylor and Wheatly, 1981; Wheatly and Taylor, 1981; Johnson and Uglow, 1987; Swain *et al.*, 1987).

Spoek (1974) demonstrated that untethered (presumably unstressed) inactive lobsters (*Homarus gammarus*) were capable

of showing a high level of respiratory independence in contrast to the results from studies using tethered animals (presumably stressed). Experiments with the crab *Carcinus maenas* demonstrated that the level of activity or state of disturbance determined the degree of respiratory independence with the $\dot{M}O_2$ of inactive animals independent of P_IO_2 down to 60 Torr (Taylor, 1976). *H. gammarus* (Spoek, 1974), *C. maenas* (Taylor, 1976), and *Austropotamobius pallipes* (Wheatly and Taylor, 1981), maintained their $\dot{M}O_2$ by increasing branchial ventilation as P_IO_2 declined, and when P_{CRIT} was reached branchial ventilation declined in all three. Temperature also influences $\dot{M}O_2$, and the $\dot{M}O_2$ of *Cherax tenuimanus* declined significantly when the P_IO_2 fell below 51 Torr at 22°C, and below 66 Torr at 26°C (Villarreal, 1990). The crab, *Carcinus maenas*, was subjected to reducing oxygen levels in 6°C, 12°C and 17°C water, and could tolerate a lower P_IO_2 in the colder water before emerging (Taylor *et al.*, 1973).

These studies suggest that general activity, stress and temperature, which all influence metabolic rate and energy requirements, in turn determine P_{CRIT}, the point at which the rate of oxygen uptake by the animal is no longer independent of the P_IO_2.

In term of crayfish ecology, the P_{CRIT} determines the conditions of water oxygenation which become detrimental to the animal's survival, and which may result in pressure to move to water which has a higher oxygen content or to emerge from the water altogether and resort to aerial respiration. Physiologically it indicates that point at which the PO_2 gradient from the medium to the mitochondrium is the lowest necessary to maintain a settled metabolism without resorting to anaerobic energy sources.

P. zealandicus can be found in a variety of aquatic habitats, but little is known of the limits these may impose on the animal's respiration. The distribution within a lake or stream may be limited by respiratory needs, among other factors. In this chapter the effect of hypoxia on crayfish will be explored, and the P_IO_2 at which $\dot{M}O_2$ is compromised will be measured, to gain a measure of the tolerance of this crayfish to reduced oxygen availability.

II MATERIALS AND METHODS

(1) Collection of animals

Crayfish of both sexes were collected from their burrows in a little stream flowing into Lake Georgina. Ovigerous females and animals with missing chelae were returned to the stream, the rest were taken to the aquarium room at the Zoology Department. This was maintained at 15 ± 1°C with a 12 hour day 12 hour night light cycle. The animals were kept in 70 cm x 40 cm x 50 cm deep tanks supplied with fresh bore water and surplus refuges made from plastic tubing. The animals were not fed for 48 hours before an experiment and were judged to be at the intermoult stage (stage C) of the moult cycle.

When the crayfish were being collected, water samples were taken from the ends of undisturbed crayfish burrows with a long piece of poly-ethylene tubing, of 3.2 mm ID, on a 50 ml syringe. The samples were immediately measured with a portable Beckman Fieldlab Oxygen Analyzer as % saturation relative to a surface sample from the middle of the stream. The water temperature was also recorded.

(2) Experimental protocol

Crayfish were put into individual respirometers between 10 am and 11 am and these were covered with black polythene. The respirometer, described in Titulaer (1991), was in a 15°C water bath and had oxygenated water circulating through it. After ten hours settling the respirometer was closed. The PO_2 in the respirometer was measured with a Strathkelvin 1302 oxygen electrode, inserted into the respirometer through a rubber bung. The electrode was connected to a Strathkelvin 781B oxygen meter and BBC SE120 Chart Recorder. The chart recorder provided an immediate and permanent visual record of the PO_2 and the rate of oxygen depletion in the respirometer.

In 18 trials the hypoxia was produced by crayfish consuming the oxygen from an initial condition of normoxia. The PO_2 within the respirometer was recorded at intervals of 30 minutes. The $\dot{M}O_2$ of the crayfish was calculated for each interval using the equation for oxygen consumption in water

from Chapter 4. The mean PO_2 within the respirometer during the interval was calculated as the mean of the initial and final PO_2 of the interval. As hypoxia increased the slope of the plot changed indicating that the rate of oxygen depletion was changing, and the intervals for recording the PO_2 were reduced to 15 or 10 minutes to maintain the precision of the estimate of $\dot{M}O_2$ and the mean PO_2 over the interval.

In seven additional trials, the PO_2 in the respirometer was reduced to 60 Torr, before the respirometer was closed, by introducing water which had been deoxygenated by bubbling nitrogen through it. In these seven trials the PO_2 within the respirometer was recorded at shorter intervals of 10 and 5 minutes.

The experiments were terminated when the chart recorder indicated that the rate of oxygen depletion was almost zero because the crayfish were no longer extracting oxygen from the water within the respirometer.

(3) Haemolymph samples

When the experiment ended the crayfish were immediately removed from the respirometer and a post-branchial haemolymph sample was taken for analysis. The crayfish haemolymph was removed from the pericardial sinus with a chilled 1 ml syringe, in the same manner as in Chapter 5. From this sample two 20 µl sub-samples were taken for two C_aCO_2 measurements in a Cameron cell, and 250 µl was used in an AVL Blood Gas Analyzer to measure P_aO_2 and pH_a. The remainder was immediately put into an Eppendorf tube, quick-frozen in liquid nitrogen, and then stored at -80°C for [lactate] measurement in a YSI 23L lactate analyzer at the end of the experiment. All these processes are described in Chapter 5.

(4) Determining P_{CRIT} with a computer program.

Taylor (1976), Herreid (1980), Wheatly and Taylor (1981) and Villarreal (1990), indicate that for many Crustacea the $\dot{M}O_2$ in response to declining oxygen availability has two distinct phases, one where the $\dot{M}O_2$ remains more or less constant while the P_IO_2 declines, and the other where the $\dot{M}O_2$ is declining as the P_IO_2 declines, with an intervening period

where the $\dot{M}O_2$ changes from one phase to the other. While a distinct change point does not necessarily exist, the change from regulating to conforming, or P_{CRIT}, is often considered to be the intersection of the regression lines from these two phases (Nickerson *et al.*, 1989; Yeager and Ultsch, 1989).

The best-fit regression lines, which minimise residual errors, and the intersection of the two regression lines were calculated with a BASIC computer program from Yeager and Ultsch (1989). The program also calculates the transition point between the regression lines, which is the abscissa value of the midpoint between the endpoint of one regression line and the adjacent endpoint of the other regression line.

The program was modified so that the division of the data could be forced, producing regression lines different from the least residual errors regression lines (Appendix D). Data points near the change from regulating to conforming (point 4 in Figure 6.1) may be included in either regression line. The outcome may be influenced by $\dot{M}O_2$ measurements at a high P_IO_2 altering the slope of the regression line describing the regulated $\dot{M}O_2$, resulting in a least residual errors solution not descriptive of the events.

In Figure 6.1A (using the method of Yeager and Ultsch 1989) the least residual errors solution had four points in the left and

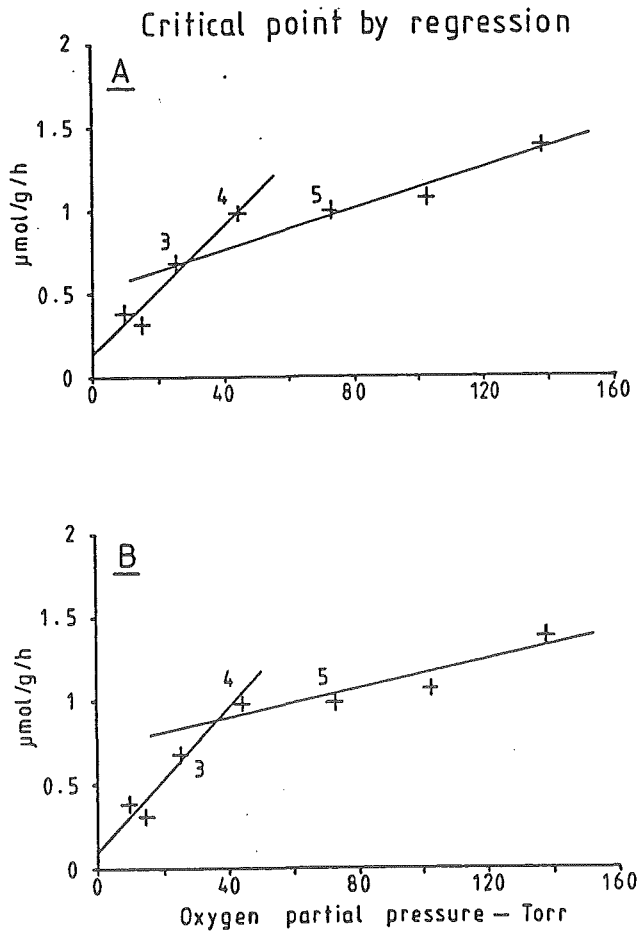


Figure 6.1 Regression lines drawn from equations provided by the program "BENTLINE", **A** is a minimum residual errors fit and **B** is a forced fit, see text for details.

three in the right regression lines. The intersection was between data points 3 and 4, the transition was between points 4 and 5, and the regressions produced a P_{CRIT} of 28.7 Torr, a transition point of 58.7 Torr, and an $\dot{M}O_2$ of 0.687 $\mu mol.g^{-1}.h^{-1}$.

In Figure 6.1B the data is divided with three points in the left and four points in the right regression lines. Both the intersection and the transition were between data points 3 and 4. The regressions produced a P_{CRIT} of 36.3 Torr, a transition point of 34.9 Torr and an $\dot{M}O_2$ of 0.882 $\mu mol.g^{-1}.h^{-1}$.

(5) Statistical methods

The $\dot{M}O_2$ of crayfish after 10 hours settling in the water, at P_{CRIT}, and at the end of the trials were analyzed with a repeated measures Anova. These results were compared with the $\dot{M}O_2$ of settled crayfish from Chapter 4, with a oneway Anova and differences identified by the Tukey-Kramer Multiple Comparisons Test. The haemolymph P_aO_2, pH_a, C_aCO_2 and [lactate] at the end of the experiment, were compared with the control values from Chapter 5 with unpaired two tailed "t" tests. The statistics for the pH are calculated directly like the other variables, and not as $[H^+]$ (Boutilier and Shelton, 1980).

The data was analyzed with the InStat version 2.04 computer package from GraphPad Software. Results are tested at the 5% level of significance and the data are presented in the table as the mean ± 1 standard error of the mean (SEM).

III RESULTS

On 10th April 1994 the water in the crayfish burrows at the collection site was found to be 95.5% ± 5.6% saturated, with a range from 79% to 100% saturation (150.9 ± 8.8 torr, n = 16), and the stream temperature was 11°C. The highest recorded temperature was 25°C, on 5th March 1990, when the stream had very low flow and the lowest temperatures were 6.5°C on 29th May 1990 and 6.2°C on 7th June 1993.

There were 18 trials in which the crayfish reduced the

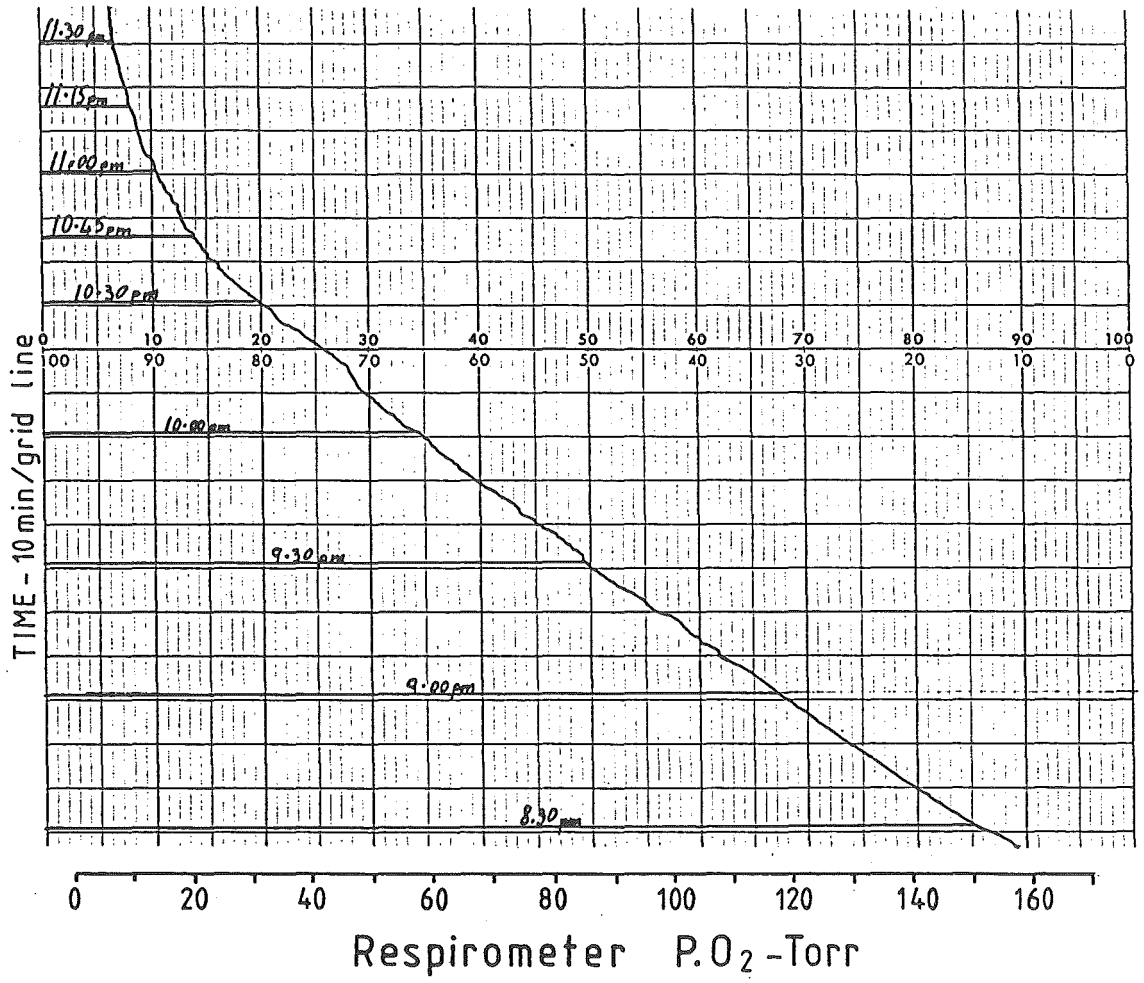


Figure 6.2 A trace of the declining PO_2 within the closed respirometer. The decline in PO_2 is almost linear down to 40 Torr, and almost zero below 10 Torr.

respirometer PO_2 from normoxia to extreme hypoxia, and 7 trials in which the crayfish reduced the respirometer PO_2 from an initial PO_2 of 60 Torr to extreme hypoxia. The later 7 trials produced results which were unworkable, and were discarded, because the $\dot{M}O_2$ values were very erratic and could not be analyzed.

The regression program produces two values for the abscissa, the intersect and the midpoint of the transition between the two regression lines, and both are summarised in Table 6.1. The intersect P_{CRIT} was 44.35 ± 3.9 Torr and the transition P_{CRIT} was 41.24 ± 2.46 Torr.

The experiments were terminated when the chart recorder indicated that the rate of oxygen depletion was approaching zero, and the mean $\dot{M}O_2$ between the last pair of data points

Table 6.1 Results from trials in which crayfish experienced progressively hypoxic water, and control values measured from crayfish in normoxic water. All results are the mean ± 1 sem.

	Normoxic water[1] 130 to 155 Torr	Progressive hypoxia 155 to 8 Torr
Settling hrs	8	10
Weight gm		32.11 ± 2.07 (18)
Initial PO_2 Torr		132.19 ± 1.79 (18)
$\dot{M}O_2$ µmol/g/h	1.092 ± 0.05 (46)	1.317 ± 0.073 (18) # *
P_{CRIT} $\dot{M}O_2$ µmol/g/h		1.065 ± 0.055 (17) #
P_{CRIT} Intersect PO_2 Torr		44.35 ± 3.9 (17)
P_{CRIT} Transition PO_2 Torr		41.24 ± 2.46 (17)
Minutes before P_{CRIT}		96.29 ± 8.47 (17)
Minutes after P_{CRIT}		51.88 ± 2.44 (17)
Lowest $\dot{M}O_2$ µmol/g/h		0.265 ± 0.016 (17) #
Lowest PO_2 Torr		8.77 ± 0.47 (15)
		50 min. hypoxia below P_{CRIT}
pH_a	7.645 ± 0.024 (29)	7.656 ± 0.027 (15)
P_aO_2 Torr	62.24 ± 4.67 (29)	11.25 ± 1.30 (13) ***
C_aCO_2 mmol/l	12.46 ± 0.48 (29)	12.11 ± 0.72 (14)
Lactate mmol/l	0.12 ± 0.02 (29)	1.3 ± 0.22 (15) ***

Values are the mean ± 1 standard error, n value in ( )
# $P < 0.0001$, extremely significant difference between settled, P_{CRIT} and hypoxic $\dot{M}O_2$.
* $P < 0.05$, significant difference from $\dot{M}O_2$ in normoxic water.
*** $P < 0.0001$, extremely significant difference from control value in normoxic water.
[1] $\dot{M}O_2$ from Chapter 4; haemolymph pH_a, P_aO_2, C_aCO_2 and [lactate] from Chapter 5 controls.

was 0.265 ± 0.016 µmol.g^{-1}.h^{-1}. The mean duration of the trials beyond their calculated P_{CRIT} was 51.9 ± 2.44 minutes.

The $\dot{M}O_2$ from crayfish which had settled for 10 hours, the $\dot{M}O_2$ at P_{CRIT} and the $\dot{M}O_2$ recorded at the end of the trials were significantly different ($F = 174.06$, $P < 0.0001$).

The crayfish showed a significant 20% decline in $\dot{M}O_2$ from 1.32 µmol.g^{-1}.h^{-1} to 1.07 µmol.g^{-1}.h^{-1}, with a 66% decline in PO_2 from 132 Torr to P_{CRIT} between 40 Torr and 45 Torr. Subsequently the $\dot{M}O_2$ declined from 1.07 µmol.g^{-1}.h^{-1} at P_{CRIT} to 0.26 µmol.g^{-1}.h^{-1} or 25% of the $\dot{M}O_2$ at P_{CRIT}, with a decline in PO_2 down to 9 Torr or 22.5% to 20% of the PO_2 at P_{CRIT}.

There was no significant difference between the control

pH_a and the experimental pH_a ($t = 0.3002$, 42 df., $P = 0.7655$), or the control C_aCO_2 and the experimental C_aCO_2 ($t = 0.4130$, 41 df., $P = 0.6817$).

There were extremely significant differences between the control [lactate] and the [lactate] after hypoxia ($t = 7.363$, 42 df., $P < 0.0001$), and between the control P_aO_2 and the P_aO_2 after hypoxia ($t = 5.864$, 40 df., $P < 0.0001$).

During the 50 minutes of compromised oxygen uptake there were no significant changes in the haemolymph pH or C_aCO_2. The P_aO_2 changed significantly from 62 Torr to 11.25 Torr, and there was a significant ten fold increase in [lactate] from 0.12 $mmol.l^{-1}$ up to 1.3 $mmol.l^{-1}$. The lowest $\dot{M}O_2$ was recorded during the last sampling interval; there were no measurements of zero oxygen uptake.

A paired two tailed "t" test between the final mean respirometer PO_2 of 8.77 Torr, recorded at the end of the last sampling interval, and the haemolymph P_aO_2 of 11.25 Torr recorded from the crayfish immediately at the end of the experiment, indicated that the difference was not significant ($t = 1.538$, 12 df., $P = 0.1500$).

It took crayfish an average of 96 minutes to reduce the PO_2 in the respirometer to P_{CRIT}, and the crayfish were in the respirometer for another 52 minutes of compromised oxygen uptake before the chart recorder indicated that the rate of oxygen depletion was approaching zero. During this time the [lactate] increased significantly from 0.12 (control from Chapter 5) to 1.3 $mmol.l^{-1}$.

During the 52 minutes hypoxia after P_{CRIT} had been reached the mean oxygen deficit was approximately 0.4 $\mu mol.g^{-1}$, or a total deficit of 12.8 µmol for a 32 g crayfish. In aerobic metabolism the quantity of energy released by 1 mole oxygen is equivalent to 6 mole lactic acid in anaerobic metabolism (Dejours, 1981). If the energy requirements were to remain the same during this period, then the 12.8 µmol oxygen deficit would result in the production of 76.8 µmol lactate. In a 32 g crayfish with 4 ml of haemolymph and a haemolymph [lactate] of 1.3 $mmol.l^{-1}$ there would be 5.2 µmol lactate in the haemolymph, and the remaining 71.6 µmol lactate would be in the remaining tissues, with a total weight of about 28 g,

including the carapace. This is about 2.56 $\mu mol.g^{-1}$ lactate, brought about by the period of oxygen shortage.

IV DISCUSSION

Anaerobic metabolism is employed when energy needs exceed the supply of aerobic energy, or when there is insufficient oxygen available. These two conditions are different situations for the same phenomenon, where the energy needed is greater than the availability of aerobic energy. In the first case the activity level is very high, and in the second case the oxygen availability is very low. The transition point, called P_{CRIT}, is the critical oxygen tension below which the animal's $\dot{M}O_2$ becomes dependent upon P_IO_2 (Herreid (1980), and is dependent on all extraneous factors which affect $\dot{M}O_2$. This means that oxygen availability will become limiting at moderate to high PO_2 in situations of high energy demand.

Taylor (1976) pointed out that "... the degree of respiratory independence shown by *Carcinus maenas* under conditions of declining oxygen tension is dependent on the animal's level of activity". Mauro and Thompson (1984) considered *Procambarus clarki* (*sic*) to be an oxyconformer in response to progressive hypoxia from 140 Torr to 15 Torr at 22°C. However the crayfish were only allowed 30 minutes settling time. The initial $\dot{V}O_2$ of 144 $\mu l.g^{-1}.h^{-1}$ ($\dot{M}O_2$ = 6.4 $\mu mol.g^{-1}.h^{-1}$) suggests that this animal had not settled at all. (This can be contrasted with crustacean oxygen consumption values in Table 4.2). Likewise Moshiri *et al.* (1970) concluded that *Pacifastacus leniusculus* was a typical conformer, but they did not ensure that their crayfish were adequately settled, and increased the stress further by using a mask on the crayfish.

P. planifrons and *P. zealandicus* were found to be more active at night (Devcich, 1979; Quilter, 1975), and the data from Chapter 3 indicate that *P. zealandicus* emerge from the water more often at night. In Chapter 5 it was found that crayfish out of the water had a haemolymph pH which was 0.1

units more alkaline in the evening than in the morning. In Chapter 8 it will be shown that control crayfish settled in water have a higher heart and scaphognathite rate in the evening than the morning. A diel rhythm of ventilatory requirements and pH (Sakakibara *et al.*, 1987) and heart rate (Pollard and Larimer, 1977) have been shown in other crayfish species also.

Massabuau *et al.* (1984) found that the ventilatory requirement of *Astacus leptodactylus* was higher in the evening than in the morning. Sakakibara *et al.* (1987) argue that this was to adjust the haemolymph pH by ventilatory control of haemolymph PCO_2, and found that the haemolymph pH of *A. leptodactylus* was 0.1 units more alkaline at dusk than at dawn. It was suggested that the alkalosis was linked to an increase in metabolism, inducing a Bohr effect when oxygen demand was increasing (Sakakibara et al., 1987).

In the experimental protocol employed here, $\dot{M}O_2$ recording was started between 8 pm and 9 pm after 10 hours settling, and the initial $\dot{M}O_2$ was found to be 20 % higher than the settled $\dot{M}O_2$ reported in Chapter 4. This suggests that it cannot be assumed that crayfish settled in the evening had the lowest possible metabolic rate. It is possible that in the morning, when the crayfish has reduced its metabolism and $\dot{M}O_2$ to lower daytime levels the P_{CRIT} will also be lower. This would suggest that when it is pertinent for the crayfish to remain inactive and hidden during the day it is also least sensitive to hypoxia.

During hypoxia the P_aO_2 in *Carcinus maenas* was between 8 and 17 Torr. This low P_aO_2 was accompanied by increased *in vivo* haemocyanin oxygen affinity maintaining the differences between pre and post branchial oxygen concentration, and this was almost fully accounted for by the combined effect of elevated pH, urate and lactate (Lallier and Truchot, 1989). Increases in haemolymph lactate concentration have been found to increase haemolymph oxygen affinity in the crayfish *A. pallipes* (7.2 $mmol.l^{-1}$, Morris *et al.*, 1986c), and in the crabs *Callinectes sapidus* (10 $mmol.l^{-1}$, Booth *et al.*, 1982; Johnson *et al.*, 1984), *Carcinus maenas* and *Cancer pagurus* (15 $mmol.l^{-1}$, Truchot, 1980).

Table 6.2 Values from *A. pallipes* in normoxic water, and at 63 Torr and 36 Torr, above and below the P_{CRIT} of 40 Torr, during progressive hypoxia lasting 12 hours (Wheatly and Taylor 1981).

	Normoxic water 145 Torr	* Hypoxic water * 63 ± 1 Torr	36 ± 2 Torr
$\dot{M}O_2$ µmol/g/h	0.672	0.762	0.294
P_aO_2 Torr	33 ± 5 (5)	13 ± 2 (8)*	8 ± 1 (6)*
pH_a	7.896 ± 0.024 (9)	7.964 ± 0.034 (8)	7.982 ± 0.027 (8)*
C_aCO_2 mmol/l	7.1 ± 0.4 (9)	4.9 ± 0.49 (8)*	4.23 ± 0.49 (8)*
Lactate mmol/l	0.4 ± 0.56 (6)	0.49 ± 0.03 (7)	0.97 ± 0.13 (8)*
P_aCO_2 Torr calc.	3.03 ± 0.24 (9)	1.73 ± 0.07 (8)*	1.45 ± 0.15 (8)*

Values are the mean ± 1 standard error, n value in ( )
* a result significantly different from normoxic values at $p < 0.05$

Wheatly and Taylor (1981) found that in *A. pallipes* $\dot{M}O_2$ was constant all the way down to the P_{CRIT} of 40 Torr. A hyperventilation of up to 2.8 times led to elimination of CCO_2 and a respiratory alkalosis of 0.1 unit, which was partly offset by a 2.5 times increase in [lactate] from 0.4 $mmol.l^{-1}$ to 1.0 $mmol.l^{-1}$ (Table 6.2). This increased the haemolymph oxygen affinity, reduced P_{50} from 8 Torr to 4.5 Torr, and maintained the a-v O_2 content difference.

P. zealandicus also experienced a significant change in haemolymph [lactate] from 0.12 to 1.3 $mmol.l^{-1}$. The severe hypoxia below P_{CRIT}, down to 9 Torr in 52 minutes, however, produced a smaller increase in haemolymph [lactate] than was expected by the total oxygen deficit. There are several possible causes for the difference;-

a) the lactate may have been excreted. It needs to be considered that the rate of excretion would need to be much higher than the normal rate of oxygen uptake (1 mole O_2 = 6 mole lactate, to provide the same energy).

b) the bulk of the lactate was still within the tissues which produced it. This leads to fatigue and is ultimately lethal.

c) that there were significant reductions in heart and scaphognathite rates when P_IO_2 fell below P_{CRIT} resulting in a

considerable decrease in $\dot{M}O_2$, and leading to an over-estimate of the oxygen deficit, and the expected lactate increase.

d) there may have been a general reduction in metabolic rate resulting in an over-estimate of the oxygen deficit, and the expected lactate increase.

A. pallipes subjected to progressive hypoxia experienced a drop in f_H, when P_IO_2 fell below 80 Torr, and an increase in f_R as P_IO_2 approached P_{CRIT} when it also declined (Wheatly and Taylor, 1981). *Astacopsis franklinii* and *Parastacoides tasmanicus* also experienced a decrease in f_H with declining P_IO_2, but an increase in f_R (Swain *et al*, 1987). *Carcinus maenas* reduced f_H when P_IO_2 fell, and did not change f_R (Taylor *et al.*, 1973).

In their study of the anaerobic metabolism of *Cherax destructor*, England and Baldwin (1983) found that the tail muscle, which is up to 25 % of the total body weight, had up to 15.8 µmol lactate per gram wet muscle weight. Greenaway *et al.*, (1992) found that in a resting crab, *Leptograpsus variegatus*, 17.4 % of the total body lactate was in the haemolymph, and in an exercised crab 7.6 % of the total body lactate was in the haemolymph, and they concluded that the haemolymph [lactate] did not reflect the total lactate content or the extent of the anaerobic metabolism during exercise. Elevated levels of lactate were found in the medium when *Cherax destructor* was recovering from tail-flipping exercises (Head and Baldwin, 1986). These examples indicate that the fate of the lactate acquired by the crayfish during the period of hypoxia is not certain. The lactate may have been excreted, it may still have been in the tissues, or the metabolism may have declined when P_IO_2 fell below P_{CRIT} resulting in an over-estimate of the oxygen deficit.

During the period of aerial respiration described in Chapter 5 (Table 5.1) the haemolymph P_aO_2 only fell below the P_{CRIT} of 41 Torr to 45 Torr once. This was after 12 hours in air (sample 6, which is also an early morning sample), when the haemolymph P_aO_2 was 32 Torr. This would indicate that at only one time during the period of aerial respiration was the

crayfish potentially experiencing an internal hypoxia sufficient to compromise normal aerobic metabolism.

Conclusions.

The P_{CRIT} was between 41 Torr and 45 Torr, and the $\dot{M}O_2$ at this point was 1.1 $\mu mol.g^{-1}.h^{-1}$

The initial settled $\dot{M}O_2$ was 1.3 $\mu mol.g^{-1}.h^{-1}$, and was significantly different from the $\dot{M}O_2$ at P_{CRIT} and the settled $\dot{M}O_2$ in the aquatic and aerial oxygen consumption experiment.

The crayfish are night active and the higher settled $\dot{M}O_2$ is attributed to the crayfish in the hypoxia experiment being sampled late at night when their metabolism is increased. It is suggested that the P_{CRIT} would be lower during the daytime.

The lactate concentration was much lower than calculated from the expected oxygen deficit experienced by the crayfish after P_{CRIT} had been reached. The lactate may have been excreted, it may still have been in the tissues, or the metabolism may have declined when P_IO_2 fell below P_{CRIT} resulting in an over-estimate of the oxygen deficit.

During aerial respiration crayfish with a P_aO_2 equal or higher than the P_{CRIT} between 41 Torr and 45 Torr were not experiencing sufficient hypoxia to compromise $\dot{M}O_2$.

CHAPTER 7

OXYGEN CAPACITY AND pH BUFFERING OF THE HAEMOLYMPH *IN VITRO*

I INTRODUCTION

(1) Oxygen Equilibrium Curve

In Chapter 4 the oxygen consumption of *P. zealandicus* in water and in air were discussed, and haemolymph changes during aerial respiration were explored in Chapter 5. Respiration is the process of removing O_2 from the external environment and supplying it to the tissues, and removing the CO_2 produced by the tissues and disposing of it in the external environment. Consequently the concentrations of O_2 and CO_2 in the haemolymph are continually changing.

The capacities of water and haemolymph for CO_2 are about thirty times that for O_2, and the transport and disposal of CO_2 can be managed adequately by dissolving it. If an animal such as a crayfish were to rely on the oxygen dissolved in the haemolymph to supply its tissues then it would need a very large oxygen transport system, or there would be severe limitations on aerobic metabolism. Although there are many organisms which only transfer oxygen in solution, most of these are small. The majority of large animals have oxygen carrying proteins either in their circulating fluid or within special cells, a haemoglobin or haemoglobin type protein in many animals including the vertebrates, and a haemocyanin in many invertebrates. The Class Malacostraca, which includes crayfish, all have haemocyanin as the carrier molecule.

Oxygen is carried by crustacean haemolymph in two ways:

1) in solution, and

2) bound to a haemocyanin carrier protein.

The quantity of oxygen which is in solution in the haemolymph is proportional to the partial pressure of oxygen and the solubility, α, which is dependent on the ionic strength of the solution.

Oxygen binds to the haemocyanin proteins in an equilibrium relationship dependent on the quantity of dissolved oxygen, which is proportional to the partial pressure of oxygen. The model by Hill (1910) suggested that although the proteins were not necessarily monomers, there was no scope for cooperativity, but it has been found that as the dissolved oxygen content changes there is a change in the oxygen-haemocyanin binding affinity of many carrier proteins.

The oxygen carriers in crustacean haemocyanin appear to be a group of similar proteins of 70k to 100k Daltons which combine in a variety of ways to form dimers, hexamers and dodecamers in the crayfish *Cherax destructor* (Murray and Jeffrey, 1974; Jeffrey *et al.*, 1976; Jeffrey, 1979; Jeffrey and Treacy, 1980; Marlborough *et al.*, 1981), *Astacus astacus* (Rogala and Gondko, 1981) and in the semi-terrestrial crab *Ocypode quadrata* (Johnson, 1987). The formation of these very large molecules is thought to be crucial to osmoregulation as a pool of monomers would exert considerable osmotic pressure.

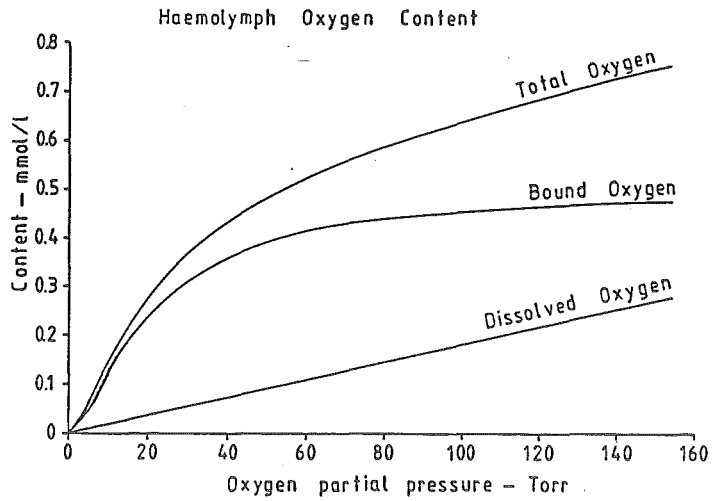


Figure 7.1 Graphs of the change in oxygen content with oxygen partial pressure for dissolved oxygen, oxygen bound to a carrier protein, and the sum of both dissolved and bound oxygen. In crustacea the dissolved part is often a large proportion of the total content. When the carrier protein is near saturated, where the PO_2 > 80 Torr in the figure, the slope of the curve of total oxygen content approaches that of dissolved oxygen content.

The Hill (1910) equation is, $y = 100(kx^n/1+kx^n)$, where y is the percent H_{cy} oxygenated, k is a constant and x is the oxygen pressure, or $y = kp^n/1+kp^n$ (Larimer and Riggs, 1964), where y is the degree (proportion) oxygenated and p is the oxygen pressure. According to Hill (1910) n represents the aggregate of n molecules of Hb in Hb_n. The simple model (see

Appendix B): $nO_2 + H_n \leftrightharpoons H_nO_{2n}$ is adequate for calculating the behaviour of a simple carrier molecule with the equilibrium constant expressed as $K = [H_nO_{2n}]/[O_2]^n[H_n]$, where H is H_b or H_{cy}. Many studies indicate, however, that as PO_2 increases the Hill coefficient (n) changes, indicating that as H_{cy} becomes more saturated k and n increase, but it is possible to establish an n value about P_{50}, which many experimenters use for comparisons and this is described as n_{50} (Morris *et al.*, 1985; Johnson, 1987; Morris, Taylor and Bridges, 1988; Greenaway *et al.*, 1992).

It would appear that the affinity of all the oxygen binding sites available in the macro-molecule is not fixed, but that as oxygen molecules attach to the haemocyanin the affinity of the remaining sites is altered. This change in affinity with oxygenation is called cooperativity. Jokumsen and Weber (1982) working with *Pagurus bernhardus*, a hermit crab, and Larimer and Riggs (1964), working with the crayfish *Procambarus simulans*, found that the n value, a measure of cooperativity, changed with the degree of oxygenation.

The shape of the $H_{cy}O \leftrightharpoons H_{cy}$ saturation curve, or oxygen equilibrium curve, changes with changes in the haemolymph chemistry. These changes, described as right or left shifts of the equilibrium curve, are brought about by changes in pH, PCO_2, temperature (Rutledge, 1981; Morris and Bridges, 1989), lactate (Truchot, 1980; Booth *et al.*, 1982; Johnson *et al.*, 1984; Taylor *et al.*, 1985; Morris *et al.*, 1986c), urate (Morris *et al.*, 1985; Lallier and Truchot, 1989), Ca^{+2} (Morris *et al.*, 1986c, 1987; McMahon, 1988), and other modulators (Morris *et al.*, 1986a; Morris and McMahon, 1989), which alter the affinity of the protein for oxygen. If the affinity is too high the oxygen will not be released at the tissues where it is required, and if the affinity is too low insufficient oxygen will be picked up at the respiratory surface.

Two common methods of comparison are:

1) the change in n_{50}, the n value at 50 % saturation, with a change in pH, and

2) the change in $logP_{50}$, the $logPO_2$ at which the haemocyanin is 50 % saturated, with pH, and which is known as the Bohr effect (Torrance and Lenfant, 1969).

A high affinity would mean that the oxygen is bound at lower oxygen partial pressures, ie the oxygen equilibrium curve is shifted to the left.

The shape of the oxygen equilibrium curve shows quite clearly that a large proportion of the oxygen carrying capacity is filled at a low PO_2 and over a narrow PO_2 range. This means that at low environmental PO_2 the oxygen can be easily taken up at the gills, and that a large portion of the carrying capacity is quickly filled. From studies of air-breathing, water-breathing and bimodal fish it appears that an increase in the oxygen carrying capacity of the blood generally accompanies the air-breathing habit, and there is a tendency for a lower blood oxygen affinity in the presence of increased oxygen availability, (Johansen *et al.*, 1978).

(2) Bicarbonate Buffering

The process of aerobic respiration produces CO_2, and changes in respiration may alter the haemolymph CO_2 content. This can have several effects:

1) activity produces an increase in demand for O_2 and results in more CO_2 being produced, increasing haemolymph CCO_2 and $[H^+]$, producing a drop in pH and a respiratory acidosis,

2) strenuous activity leading to hyperventilation results in CO_2 being washed out of the haemolymph, decreasing the CCO_2 and $[H^+]$, producing a rise in pH and a respiratory alkalosis,

3) changes in haemolymph PCO_2 and pH alter the oxygen affinity. An increase in PCO_2 lowers the pH and moves the equilibrium curve to the right, decreasing oxygen affinity, and a decrease in PCO_2 has the reverse effect. This is known as the Bohr effect.

The elevated ventilation initiated by activity or hypoxia increases the supply of oxygen to the respiratory surface and produces an associated decrease in haemolymph PCO_2, which in turn increases oxygen affinity through a negative normal Bohr shift (McMahon, 1988). Metabolic acids can produce similar effects.

Physiological processes depend on a supply of energy and a stable physical and chemical environment within the cells

and the haemolymph. The capacity to carry sufficient oxygen and vary this in response to changes in demand, and the ability to buffer the haemolymph pH are both very important. In this chapter the haemolymph oxygen carrying capacity, and its response to pH and temperature will be investigated, along with the ability of the crayfish to buffer haemolymph pH as PCO_2 changes. The model of oxygen equilibrium curve proposed by Hill (1910) will be manipulated to gain a measure of the effect of n_{50}, k, and $\alpha_{PLASMA}O_2$, oxygen solubility in plasma, on the P_{50} and the P_{50}content. The oxygen carrying capacity and the buffering characteristics will assist in the interpretation of the haemolymph changes experienced by the crayfish during aerial respiration.

II MATERIALS AND METHODS

(1) Collection of animals

Crayfish of both sexes were collected from a little stream flowing into Lake Georgina, and ovigerous females and animals with missing chelae were returned to the stream. The stream temperature was recorded at the collection site. The crayfish were kept in the Zoology Department aquarium room in 70 cm x 40 cm x 50 cm deep tanks supplied with bore water and surplus refuges made from plastic tubing, with a 12 hour day 12 hour night light cycle and at 15 ± 1°C. The experimental animals were judged to be at the intermoult stage (stage C) of the moult cycle.

(2) Oxygen Equilibrium Curve

The oxygen equilibrium curve was established from haemolymph which had been clotted, centrifuged, mixed and oxygenated in a tonometer, and sampled to measure the oxygen content in a Tucker cell and the pH and PO_2 in an AVL blood gas analyzer.

A chilled hypodermic syringe was used to remove 0.6 ml to 1.0 ml haemolymph from the pericardial region of 20 to 22 crayfish, which were then returned to a recovery tank. The haemolymph was put into 1 ml Eppendorf tubes and left to clot

on ice. The clot was broken down, first with a glass rod, then by passing it through a fine hypodermic needle, and the haemolymph was then centrifuged in an Eppendorf model 3200 centrifuge for 5 minutes. The haemolymph was pooled in a tonometer and a 300 µl sample was immediately put into an Eppendorf tube, quick-frozen in liquid nitrogen, and stored at -80°C for subsequent [lactate] measurement in a YSI 23L lactate analyzer.

The tonometer was maintained at 15°C ± 0.1°C, and low volumes of humidified gases at 15°C ± 0.1°C ensured steady changes in PO_2 and oxygen content. PCO_2 was used to maintain and alter the haemolymph pH. The gas mix was produced by a Wösthoff Digamix type 2M 201/a-F gas mixing pump from cylinders of 100% oxygen, 100% nitrogen, and a 5% CO_2 - 95% nitrogen mix. As the PO_2 changed 400 µl samples of haemolymph were removed from the tonometer and a 100µl sub-sample of this was used to measure total oxygen content in a Tucker cell, the remainder being used in the AVL Blood Gas Analyzer to measure the PO_2 and pH.

The Tucker cell, with a fluid volume of 1 ml, contained a solution with 6g/l KCN and 3g/l saponin, and was operated at 37°C ± 0.1°C to ensure the rapid release of the oxygen bound to the haemocyanin (Bridges *et al.*, 1979). Changes in PO_2 were recorded with a Strathkelvin 1302 oxygen electrode, a Strathkelvin 781b oxygen meter and a Beckman recorder. The electrode was calibrated with Na_2SO_3 solution and air-saturated water at 37°C. The calibration of the oxygen electrode and the Beckman recorder were checked by measuring the deflection produced by 100µl of water saturated with pure O_2 at 37°C. From this the deflection on the plotter per Torr change in the Tucker cell could be calculated.

The AVL blood gas analyzer was operated at 15°C ± 0.1°C. The oxygen electrode was calibrated with humidified oxygen-free nitrogen and air-saturated water at 15°C and the pH was checked with Radiometer precision buffer solutions, S1500 and S1510, which read 6.900 and 7.445 respectively at 15°C.

The haemolymph oxygen content was calculated by the method of Bridges *et al.* (1979) which takes into account the displacement of a portion of the Tucker cell volume by the

100 µl haemolymph sample.

Two trials were conducted with haemolymph from crayfish which had been acclimated to 15°C in the aquarium room for 2 months, A with 1 % CO_2, and B with 0.5 % CO_2 (PCO_2 of 7.6 Torr and 3.8 Torr). Fresh crayfish were captured, and in the subsequent three days haemolymph from these crayfish was used in three more trials, C with 1 % CO_2, D with 0.5 % CO_2 and E with 0.25 % CO_2 (PCO_2 of 7.6 Torr, 3.8 Torr and 1.9 Torr). The fresh crayfish were collected at the end of October, when the stream temperature was 9.5°C, and these animals will be considered to be cold acclimated.

(3) Calculating the Oxygen Equilibrium Curves

It is difficult to establish a value for $\alpha_{PLASMA}O_2$ from the literature as little information is provided about the methods and calculations used. The $\alpha_{PLASMA}O_2$ for *P. zealandicus* haemolymph was estimated to be 1.8 $\mu mol.l^{-1}.Torr^{-1}$ at 15°C, using ionic strengths and osmotic pressures, between 51 % and 55 % the value of seawater, reported in Wong and Freeman (1976 a,b & c). The slope of the regression line of the total oxygen content against PO_2 for values of PO_2 above 70 Torr should not be less than the slope of $PO_2 \times \alpha_{PLASMA}O_2$.

The $H_{cy}O_2$ was calculated for all the results with:

$$H_{cy}O_2 = \text{total oxygen content} - \alpha_{PLASMA}O_2 \times PO_2 \qquad (1)$$

Regression of log.(P/(1-P)) against log.PO_2 (P is the proportion H_{cy} bound to oxygen) was calculated for oxygen saturation values between 25 % and 75 % to produce a Hill plot, and calculate the n_{50} (Bridges *et al.*, 1984; Taylor *et al.*, 1985; Bridges, 1986; Morris and Bridges, 1986, 1989; Morris *et al.*, 1986a, 1986c) and the k value (see Appendix B). Oxygen equilibrium curve calculations and regressions were calculated with the Planperfect 5.1 spreadsheet.

The proportion of $H_{cy}O_2$ can be calculated from PO_2, n and k.

$$P = k(PO_2)^n \; / \; (1 + k(PO_2)^n) \qquad (2)$$

The P_{50} and P_{50}content were calculated from:

$$P_{50} = 10^{(log1 - logk)/n} \quad (3)$$

$$P_{50}\ \text{content} = P_{50} \times \alpha_{PLASMA}O_2 + \text{max.}H_{cy}O_2 \times (0.5/\text{max.P}) \quad (4)$$

where max.P is the highest proportion $H_{cy}O_2$ calculated (equation 2), and max.$H_{cy}O_2$ is the content at max.P.

Oxygen equilibrium curves were produced with the equation:

$$\text{Content} = PO_2 \times \alpha_{PLASMA}O_2 + \text{max.}H_{cy}O_2 \times (\text{Proportion bound/max.P}) \quad (5)$$

The equilibrium constant $K = [H_nO_{2n}]/[O_2]^n[H_n]$ was calculated from:

$$k = K(\alpha_{PLASMA}O_2)^n \quad (6)$$

A series of calculations were carried out with the mathematical model of the OEC from experiment A, to determine the effect of n, k, $\alpha_{PLASMA}O_2$ and Max.$H_{cy}O_2$ on the P_{50} and the P_{50}content.

(4) Bicarbonate Buffer Line

A portion of the centrifuged haemolymph from trial E had been quick-frozen in liquid nitrogen. This was thawed and used for the determination of the bicarbonate buffer line in a tonometer at 15°C ± 0.1°C. PCO_2 was mixed at 0.1 %, 0.2 %, 0.3 %, 0.4 % 0.5 % and 1.0 % (0.76, 1.52, 2.28, 3.04, 3.8 and 7.6 Torr) by a Wösthoff Digamix type 2M 201/a-F gas mixing pump, from cylinders of 100 % oxygen, 100 % nitrogen, and a 5 % CO_2 - 95 % nitrogen mix. Low volumes of the humidified gas mix were passed through the tonometer and changes in the haemolymph PCO_2, pH and CCO_2 were measured.

The CCO_2 of a sample was measured using a 2.8 ml Cameron cell filled with 0.01N HCl, operating at 40°C to accelerate the reaction, and connected to a Radiometer PHM 84 Research pH Meter. The cell was calibrated with a 10 mmol.l^{-1} $NaHCO_3$

standard which was prepared daily. Two haemolymph samples of 20 µl were measured between two 20 µl standards. The pH and PCO_2 were measured with a Radiometer PHM 71/PHM 935 acid-base analyzer. As the PCO_2 levels were low a calibration curve was constructed on an expanded scale, with 1% CO_2 (7.6 Torr) reading 70 on the scale, and this was used to convert the experimental results.

Ball (1987) calculated the values of αCO_2 = 0.0559 $mmol.l^{-1}.Torr^{-1}$, pK_1 = 6.165 and pK_2 = 9.604, for *Paranephrops* and these were used for the buffer calculations.

Haemolymph pH and PCO_2 were used to calculate the $[HCO_3^-]$ ($mmol.l^{-1}$) with the equation:

$$[HCO_3^-] = \alpha CO_2 \times PCO_2 \times 10^{(pH - pK1)},$$

The buffer value, ß = $\Delta[HCO_3^-]/\Delta pH$, and regressions for the buffer line and for the haemolymph CCO_2 and pH were calculated with the computer package Minitab 8.2. Davenport diagrams were drawn with the CCO_2 and HCO_3^- lines and with the haemolymph pH and CCO_2 changes, from Chapter 5, recorded during 48 hours aerial respiration. The PCO_2 isopleths were calculated using the equation:

$$PCO_2 = CCO2 / \alpha CO_2 \ (1 + 10^{(pH - pK1)} \ (1 + 10^{(pH - pK2)})).$$

CCO_2 was calculated from the haemolymph pH and PCO_2 measured during 48 hours aerial respiration (Chapter 5):

$$CCO_2 = \alpha CO_2 \times PCO_2 \ (1 + 10^{(pH - pK1)} \ (1 + 10^{(pH - pK2)}))$$

The equations are derived in Appendix C.

(5) Mathematical and Statistical treatment

The mathematical model for the oxygen equilibrium curve and reactions of carbon-dioxide in solution are developed in Appendix B and Appendix C respectively, and were calculated in the Planperfect 5.1 spreadsheet. InStat version 2.04

computer package from GraphPad Software was used to calculate the regression lines. Experimental data are presented as the calculated values or the mean ± 1 standard error of the mean (SEM), where appropriate.

III RESULTS

(1) Oxygen Equilibrium Curve

The results of the oxygen equilibrium curve experiments on the crayfish acclimated to the aquarium room, Group 1, and the cold adapted crayfish freshly captured from their stream habitat, Group 2, are presented in Table 7.1. In the table, the maximum calculated haemocyanin-oxygen content recorded in an experiment is "Maximum bound" (µmol/l), and the calculated haemocyanin saturation at this point is "Maximum $H_{cy}O_2$ %". The reaction constant K comes from $K = [H_nO_{2n}]/[O_2]^n[H_n]$.

The regression of the oxygen equilibrium curve data above 70 Torr was least in experiment E, and produced a slope of 1.81e-06 (mol/l/Torr) which is close to the estimated value of 1.8 µmol/l/Torr at 15°C used for $\alpha_{PLASMA}O_2$ in all the calculations. The haemocyanin saturation in experiment E was calculated to be 97 % at a PO_2 of 133 Torr.

The calculated values for $\alpha_{PLASMA}O_2$, n, k and max.$H_{cy}O_2$ at the highest experimental PO_2 were used to produce the oxygen equilibrium curves for the five experiments, Figure 7.2, and the calculated P_{50} and P_{50}content are shown on the oxygen equilibrium curves in Figure 7.3.

In Group 1 the increase in PCO_2 from 3.8 Torr to 7.6 Torr was accompanied by a decrease in pH from 7.75 to 7.57, an increase in P_{50} from 14 Torr to 17 Torr, a small increase in n, $(=n_{50})$, and a decrease in K, the reaction equilibrium constant.

In Group 2 the increase in PCO_2 from 1.9 Torr to 7.6 Torr was accompanied by a decrease in pH from 7.95 to 7.56, an increase in P_{50} from 10 Torr to 19.5 Torr, a decrease in n, $(=n_{50})$, from 1.4 to 1.1, and a decrease in K.

The Bohr effect measured over the change in PCO_2 from 3.8 Torr to 7.6 Torr was similar in the two groups, $\phi = -0.48$ in

Table 7.1 Results of the analysis of the data from the Oxygen Equilibrium Curve experiments. Group 1 is from animals kept at 15°C in the aquarium room, Group 2 from animals freshly captured from the stream at 9.5°C.

	GROUP 1		GROUP 2		
Experiment	A	B	C	D	E
Data points N	19	18	24	22	23
PCO_2 Torr	7.6	3.8	7.6	3.8	1.9
[lactate] mmol/l	0	0.55	0.45	0.7	0.1
pH	7.57	7.747	7.562	7.772	7.956
± sem	± 0.005	± 0.011	± 0.005	± 0.004	± 0.004
Highest PO_2 Torr	142.8	148.9	146	146.7	133.5
Regression of the OEC data, PO_2 > 70 Torr, approaching $H_{cy}O_2$ saturation.					
Regression slope	3.51e-06	2.74e-06	3.18e-06	2.47e-06	1.81e-06
Regression of the data from the Hill equation: $\log.k + n.\log.PO_2 = \log.(P/(1-P))$					
Intersect, log.k	-1.4923	-1.3832	-1.4326	-1.4722	-1.3948
k	0.0322	0.0414	0.0369	0.0337	0.0403
slope, n	1.2107	1.2055	1.1115	1.2449	1.3876
Calculated results using n and k from above, and $\alpha_{PLASMA}O_2$ = 1.8 µmol/l/Torr					
P_{50} (Torr)	17.085	14.0425	19.4463	15.2252	10.1196
P_{50} content µmol/l	453.28	426.28	296.11	297.6	225.33
Reaction const., K	290155	348242	89648	477719	3773757
Maximum $H_{cy}O$ % Calc.	92.89	94.51	90.38	94.38	97.28
Maximum bound µmol/l	785	758	472	510	403
Bohr effect, $\Delta\log P_{50}/\Delta pH$		A:B =	-0.4812		
		C:D =	-0.5058		
		D:E =	-0.9641		
		C:E =	-0.7198		

the 15°C crayfish and ϕ = - 0.51 in the cold acclimated crayfish. Both groups demonstrate a similar sensitivity to ΔpH and ΔPCO_2 over the same PCO_2 range and similar pH range (pH 7.57 - 7.75 and pH 7.56 - 7.77). Haemolymph from the crayfish in Group 1 (A and B), kept at 15°C in the aquarium room, had P_{50}content (Figure 7.3) and maximum bound O_2 values (Figure 7.2) which were 50 % higher than the haemolymph from the cold acclimated crayfish in Group 2 (C, D and E).

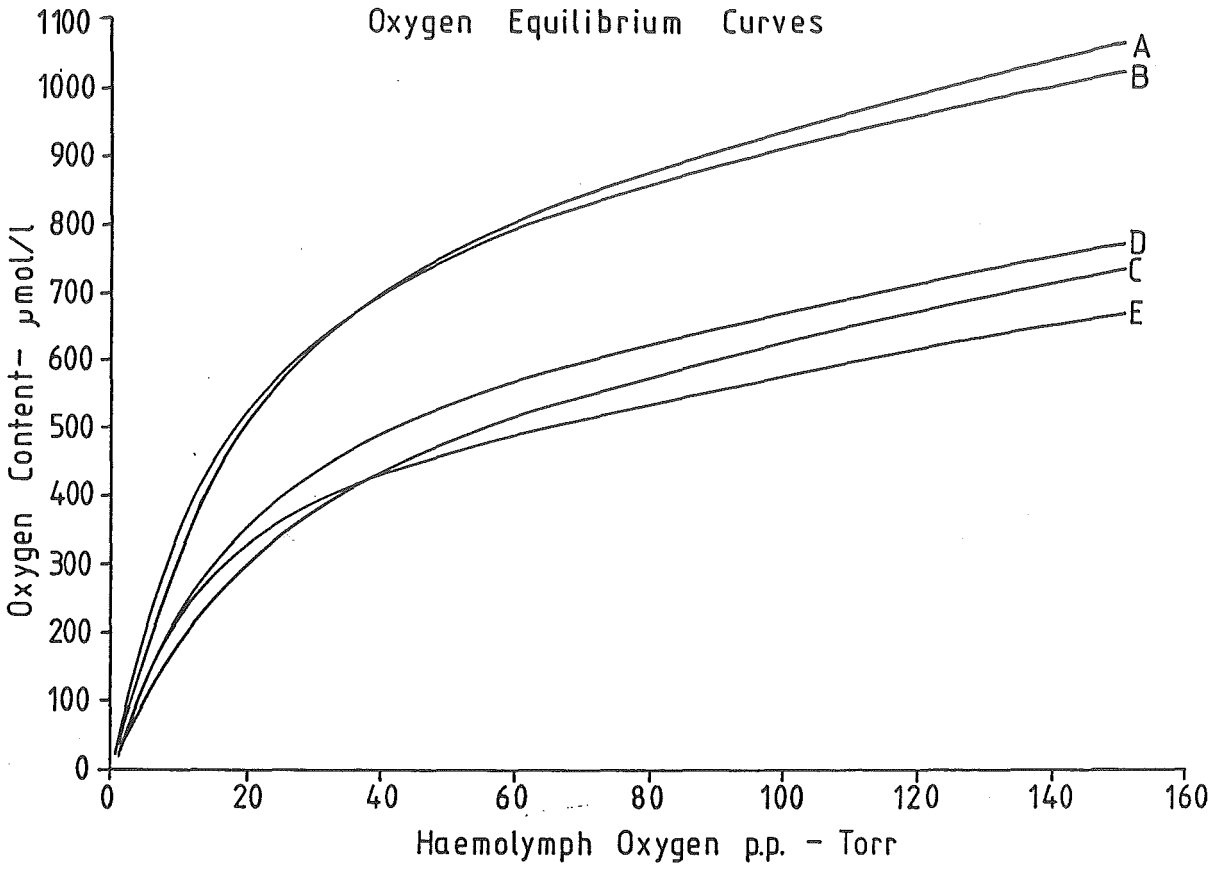


Figure 7.2 The five oxygen equilibrium curves, at 15°C, generated from the analysis presented in Table 7.1. A and B represent haemolymph from animals kept at 15°C, C,D and E from animals collected at 9.5°C.

(2) Manipulating the OEC model

A series of calculations were conducted with the oxygen equilibrium curve from experiment A. The mathematical model demonstrates that the P_{50} changes in a non-linear inverse manner with n and k, Table 7.2. As n or k increase, P_{50} decreases, ie. affinity increases, and small changes in either n or k have a very large impact on oxygen affinity. The amount of oxygen bound to haemocyanin depends on the quantity of haemocyanin or the value of n and changing the maximum quantity of oxygen bound to haemocyanin does not change the P_{50}. The P_{50}content experiences small changes with changing values of $\alpha_{PLASMA}O_2$, and larger proportional changes with the total haemocyanin oxygen content.

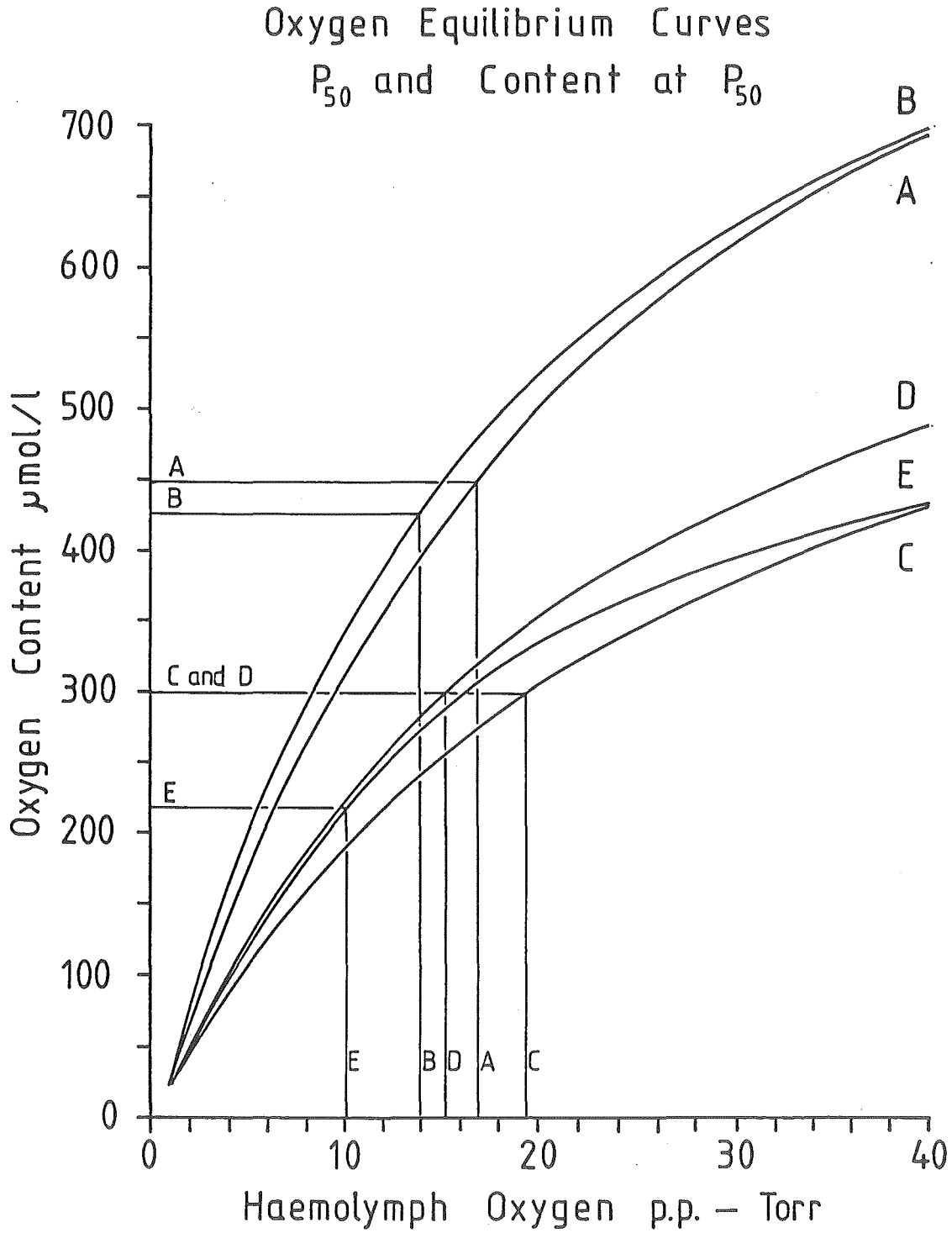


Figure 7.3 Oxygen equilibrium curves measured at 15°C, with the P_{50} and P_{50}content lines, from the data in Table 7.1. A and B from animals kept at 15°C, C, D and E from animals collected at 9.5°C.

Table 7.2 Results of calculated oxygen equilibrium curves with a variety of *n* and *k* values, and fixed $\alpha_{PLASMA}O_2$ and $Max.H_{cy}O_2$ values. The P_{50} is very sensitive and both P_{50} and P_{50}content reduce as either *n* or *k* increase.

k = 0.03 $\alpha_{PLASMA}O_2$ = 1.8 µmol/l/Torr $Max.H_{cy}O_2$ = 785 µmol/l				n = 1.2 $\alpha_{PLASMA}O_2$ = 1.8 µmol/l/Torr $Max.H_{cy}O_2$ = 785 µmol/l			
n	P_{50} Torr	P_{50}cont µmol/l	*K* Eqn const	k	P_{50} Torr	P_{50}cont µmol/l	*K* Eqn const
1.10	24.2	492	62564	0.020	26.1	490	156568
1.15	21.1	474	121215	0.025	21.6	472	195710
1.20	18.6	460	234852	0.030	18.6	460	234852
1.25	16.5	449	455020	0.035	16.3	451	273994
1.30	14.8	440	881590	0.040	14.6	444	313136
1.35	13.4	433	1708061	0.045	13.3	439	352278
1.40	12.2	427	3309327	0.050	12.1	434	391420
1.45	11.2	423	6411743	0.055	11.2	431	430562
1.50	10.4	419	12422600	0.060	10.4	428	469704
1.55	9.6	416	24068492	0.065	9.8	426	508846

The reaction equilibrium constant, *K*, also changes considerably. From the equation $k = K(\alpha_{PLASMA}O_2)^n$ it is obvious that *k* and *K* will alter linearly if $\alpha_{PLASMA}O_2$ and *n* are constant, and this is seen in Table 7.2. The less obvious relationship is the rapid change in *K* with *n*, and this occurs because $\alpha_{PLASMA}O_2$ = 1.8 µmol/l/Torr, ie 1.8 × 10^{-6} mol/l/Torr, and consequently $(\alpha_{PLASMA}O_2)^n$ is an even smaller number.

(3) Bicarbonate Buffer Line

The calculated *in vitro* haemolymph non-bicarbonate buffer value for *P. zealandicus* was - 3.82 $mmol.l^{-1}.(pH\ unit)^{-1}$. The *in vitro* haemolymph CCO_2, calculated from measured pH and PCO_2, is given by the equation, CCO_2 = 39.0 - 4.08 pH, and the measured *in vitro* haemolymph CCO_2 content is given by the equation, CCO_2 = 38.9 - 3.79 pH, Figure 7.6.

Table 7.3 summarises the haemolymph pH, CCO_2 and PCO_2 recorded in the experiment investigating the effect of aerial respiration on the crayfish (Chapter 5). The table also presents the expected CCO_2 calculated from haemolymph pH and PCO_2, and the expected PCO_2 calculated from haemolymph pH and

Table 7.3 Changes in crayfish haemolymph pH, CCO_2 and PCO_2, during settling in water, 48 hours in air, and recovery in water, from the experiment in Chapter 5. Also the expected CCO_2 calculated from the measured pH and PCO_2, and the expected PCO_2 calculated from the measured pH and CCO_2.

		Measured			Calculated	
TIME	medium	pH	CCO_2 mmol/l	PCO_2 Torr	CCO_2 mmol/l	PCO_2 Torr
10 am	water	7.630	10.71	2.67	4.55	6.29
1 pm	water	7.694	11.35	3.60	7.08	5.77
6 pm	water	7.624	10.61	2.63	4.42	6.31
8 pm	air	7.616	11.55	2.97	4.91	6.99
11 pm	air	7.653	13.81	4.11	7.38	7.69
8 am	air	7.552	15.83	6.06	8.68	11.05
8 pm	air	7.647	17.68	5.29	9.36	9.99
8 am	air	7.580	16.85	4.18	6.36	11.06
8 pm	air	7.676	18.28	5.07	9.59	9.67
10 pm	water	7.651	16.62	3.36	6.00	9.31
1 am	water	7.705	13.60	2.34	4.73	6.73
8 am	water	7.683	11.88	2.08	4.00	6.19
4 pm	water	7.739	12.18	2.57	5.61	5.58

αCO_2 = 0.0559 mmol/l/Torr pK1 = 6.165 pK2 = 9.604

CCO_2. The experimental CCO_2 vs pH data points from Table 7.3 are plotted in the Davenport diagram in Figure 7.4, and includes the experimental *in vitro* haemolymph total CO_2 content line. The experimental PCO_2 does not correspond with the CO_2 isopleths in the Davenport diagram for any of the data points.

Figure 7.5 plots experimental CCO_2 vs pH data points from Table 7.3, with the time of day and the transfer points from water to air and back to water indicated. The crayfish demonstrate a regular shift of 0.1 pH units between the morning and evening, allowing for the disturbance caused by a change in respiratory medium, with the haemolymph having a more acid pH in the morning, and a more alkaline pH in the evening.

The experimental pH and calculated CCO_2, Table 7.3, were used to plot the Davenport diagram in Figure 7.6. The figure includes the measured *in vitro* haemolymph total CO_2 content line, the calculated *in vitro* haemolymph total CO_2 content

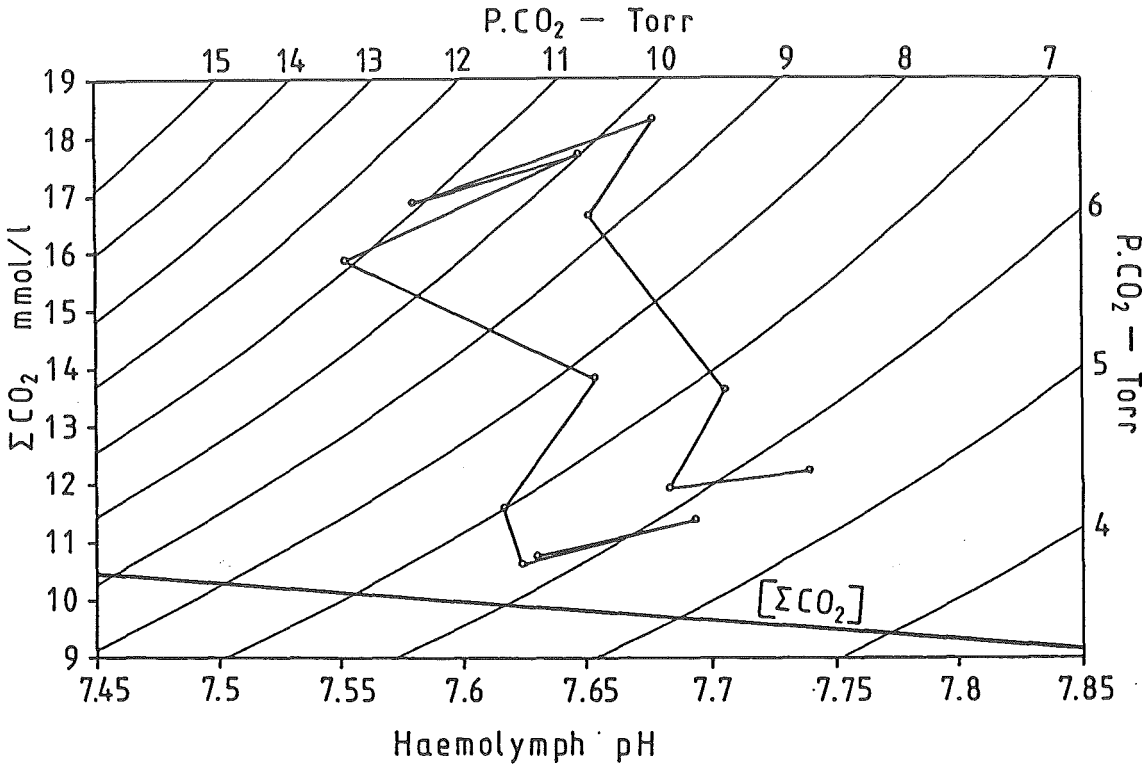


Figure 7.4 Davenport diagram with CCO_2 buffer line, for crayfish settling in water, 48 hours in air, and recovering in water. Measured PCO_2 and PCO_2 isopleths do not coincide.

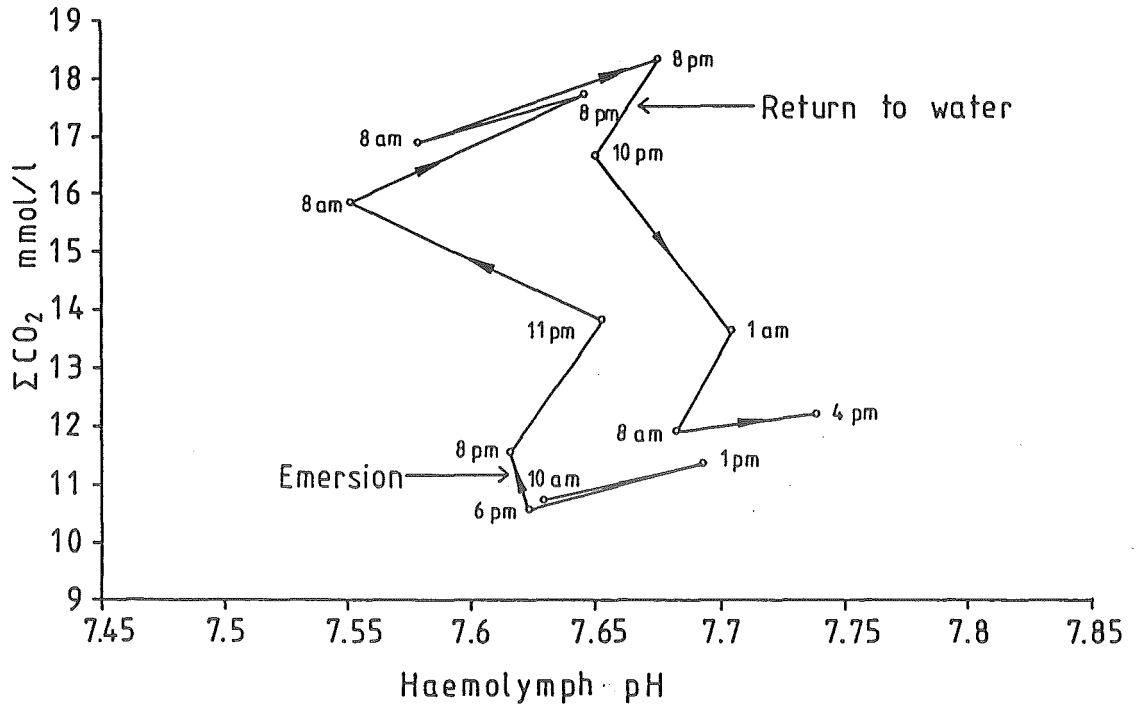


Figure 7.5 Changes in crayfish haemolymph CCO_2 with pH while settling in water, 48 hours in air, and recovery in water, with time of day included. Note the regular changes in pH between the morning and evening.

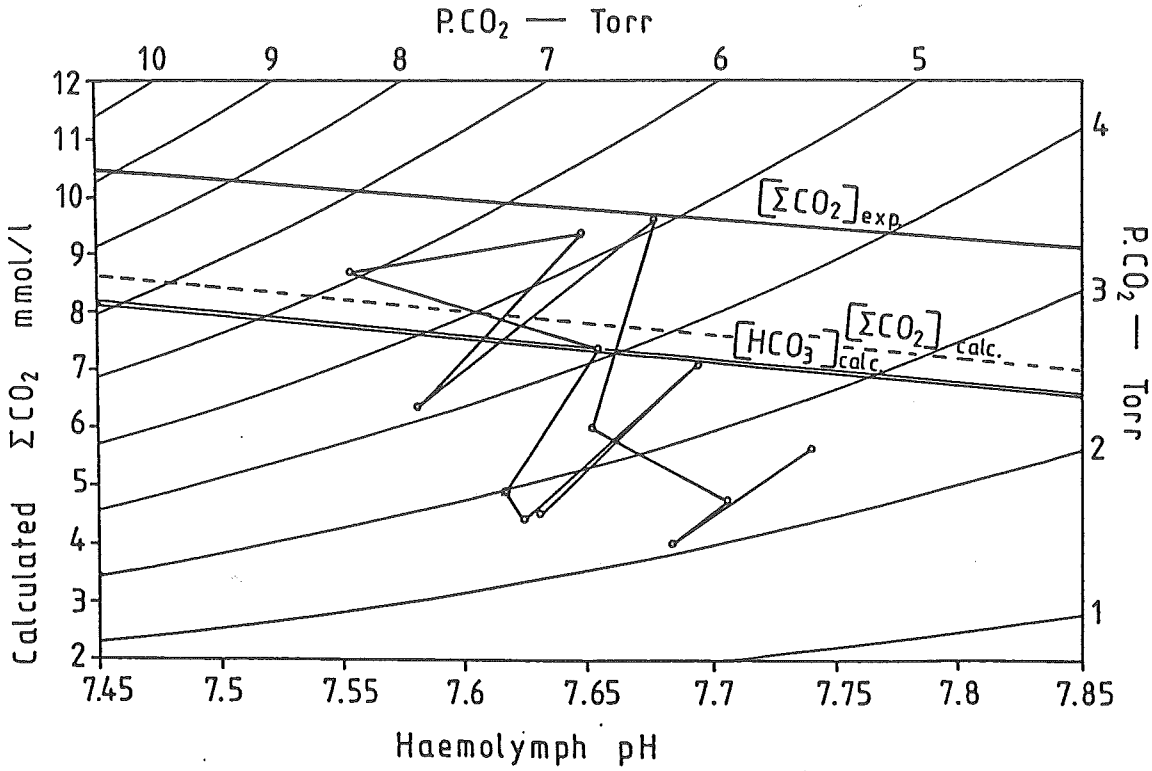


Figure 7.6 Davenport diagram, with calculated CCO_2 and measured pH values and with experimental CCO_2, calculated CCO_2 and calculated HCO_3^- buffer lines.

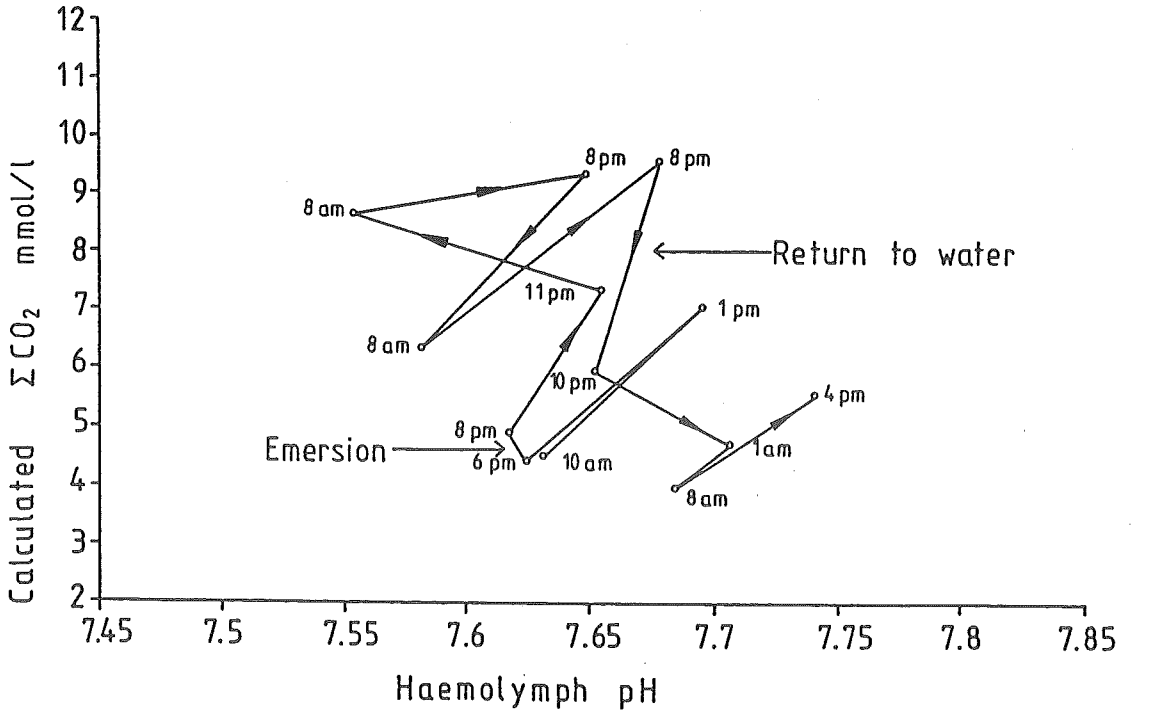


Figure 7.7 Changes in calculated haemolymph CCO_2 with pH while settling in water, 48 hours in air, and recovery in water, with time of day included. Note the regular changes in pH between the morning and evening.

line and the calculated *in vitro* haemolymph non-bicarbonate buffer line, all calculated from the measured PCO_2, CCO_2 and pH in Table 7.4. The experimental PCO_2 now corresponds with the CO_2 isopleths, but all the CCO_2 values are 4 to 10 mmol/l lower than the observed experimental values. Also the calculated *in vitro* haemolymph total CO_2 content line is 1.7 mmol/l lower than the measured *in vitro* haemolymph total CO_2 content line.

Figure 7.7 plots the measured pH and calculated CCO_2, Table 7.3, with the time of day and the transfer points from water to air and back to water indicated. The plot still indicates a regular pH shift of 0.1 units between the morning and evening.

The regression of the CCO_2 with PCO_2 produced the equation $CCO_2 = 7.724 + 0.476.PCO_2$, Figure 7.8, with a slope significantly different from zero F = 15.053, P = 0.0178, r = 0.8889 and with no significant departures from linearity.

Table 7.4 Haemolymph PCO_2, CCO_2 and pH measurements used to establish buffer capacity.

PCO_2 Torr	CCO_2 mmol/l	pH
1.30	9.17	8.12
1.48	7.74	8.03
1.65	8.64	7.90
2.70	8.81	7.83
3.48	9.15	7.69
6.77	11.10	7.54

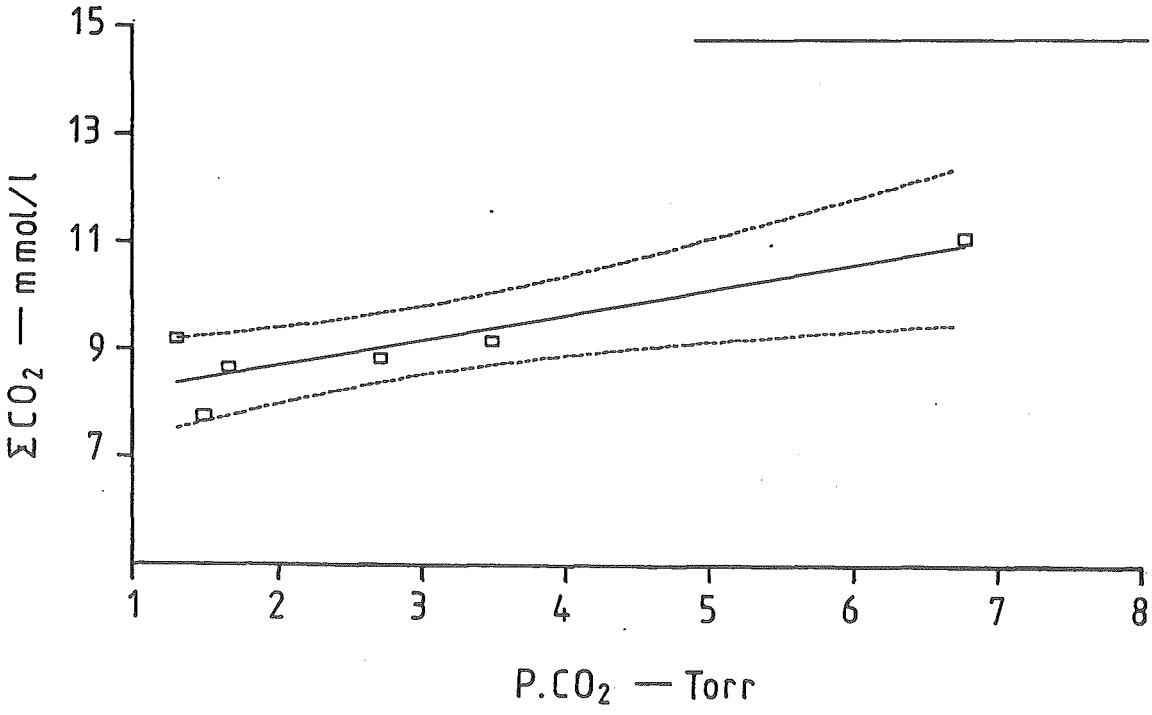


Figure 7.8 Plot of the regression of CCO_2 with PCO_2 with 95 % confidence intervals shown. The regression equation is $CCO_2 = 7.724 + 0.476.PCO_2$.

IV DISCUSSION

(1) Establishing $\alpha_{PLASMA}O_2$

The ionic strengths and osmotic pressures reported in Wong and Freeman (1976 a,b & c) between 51% and 55% the value of seawater produced a value for $\alpha_{PLASMA}O_2$ of 1.8 µmol/l/Torr at 15°C. A higher value is not possible as the least slope calculated from the regression of near-saturated haemolymph indicates a maximum possible value of 1.81 µmol/l/Torr, (Experiment E, Table 7.1, also see Appendix B). An $\alpha_{PLASMA}O_2$ of 1.5 µmol/l/Torr at 15°C for *Carcinus maenas* (Lallier and Truchot, 1989, from Truchot, 1971) suggests a plasma ion content near 130% sea-water, ($ß_{DW}O_2$ is 2.0101 and $ß_{SW}O_2$ is 1.6218 µmol/l/Torr at 15°C, Dejours, 1981). The value of 2.2 µmol/l/Torr at 10°C used by Bridges (1986, from Altman and Dittmer, 1971) for three marine crabs and a marine crayfish suggest that these Crustacea had a plasma ion content of 9 % sea-water. Booth *et al.* (1982) assumed that $\alpha_{PLASMA}O_2$ for the Blue Crab *Callinectes sapidus*, at 20°C, was 90 % of that for sea water, which would be 1.516 µmol/l/Torr ($ß_{SW}O_2$ at 20°C is 1.4823 µmol/l/Torr, Dejours, 1981). Higher values for the $\alpha_{PLASMA}O_2$ of *P. zealandicus* would imply a lower haemolymph ion content, and a much lower value would mean that $\alpha_{PLASMA}O_2$ is approaching that of sea-water, 1.6218 µmol/l/Torr at 15°C (Dejours, 1981), and a higher haemolymph ion content.

It can be seen from Table 7.5 that it is important to establish an accurate value for $\alpha_{PLASMA}O_2$ from the outset as all the subsequent calculations of n_{50}, k, P_{50}, P_{50}content and K are affected by this. In experiment E the haemocyanin oxygen saturation was calculated to be 97 % at 133 Torr, Table 7.1, and the slope of this dataset is the nearest estimate of $\alpha_{PLASMA}O_2$ and can be considered the upper limit.

The change in the oxygen equilibrium curves, and the consequent improvement in the estimate for $\alpha_{PLASMA}O_2$ is most obvious when comparing the highest PO_2, maximum $H_{cy}O_2$ % and regression slopes of the three trials in Group 2. The higher the n_{50} value, trials C to E, the lower the P_{50} and the more quickly the H_{cy} is oxygen saturated. This results in a higher $H_{cy}O_2$ % at lower PO_2, and consequently the slope for the

Table 7.5 Different values for $\alpha_{PLASMA}O_2$ produce very different values for the n_{50} and k derived from the Hill equation, and consequently a different P_{50}.

$\alpha_{PLASMA}O_2$		A	B	C	D	E
1.80e-06	k	0.0322	0.0414	0.0369	0.0337	0.0403
	slope, n_{50}	1.2107	1.2055	1.1115	1.2449	1.3876
	P_{50}	17.1	14.0	19.4	15.2	10.1
1.70e-06	k	0.0304	0.0372	0.0355	0.0327	0.0253
	slope, n_{50}	1.2225	1.2348	1.1089	1.2378	1.5188
	P_{50}	17.4	14.4	20.3	15.9	11.3
1.60e-06	k	0.0287	0.0333	0.0341	0.0316	0.0260
	slope, n_{50}	1.2341	1.2667	1.1071	1.2332	1.4703
	P_{50}	17.7	14.7	21.2	16.5	12.0

oxygen equilibrium curve at high PO_2 will be a closer approximation for $\alpha_{PLASMA}O_2$.

(2) Oxygen Equilibrium Curve

(a) The effect of temperature. The data in Table 7.1 show that a decrease in the PCO_2 increased the pH, and decreased the P_{50}. The crayfish from the aquarium room, kept at 15°C had a total and P_{50} oxygen content about 50 % higher than the crayfish from the colder water, at 9.5°C.

When the haemolymph from cold acclimated animals is experimented on at a higher temperature there is an increase in P_{50}. This was observed in *the crayfish Pacifastacus leniusculus* by Rutledge (1981). As temperature rises, pH falls (Stewart, 1981), and consequently an increase in experimental temperature will produce an effect similar to a drop in pH, resulting in an increase in P_{50}. Data from Rutledge (1981) suggests that the P_{50} values for Group 2 would be at least 1 Torr lower if the measurements had been carried out at the acclimation temperature of 9.5°C instead of the experimental temperature of 15.0°C. Data from Stewart (1981) on the change in pH with temperature ($\Delta pH/\Delta T \approx -1/60$ between 0-60°C and ph 6.5 to 7.5) would alter the P_{50} values by up to -2 Torr, increasing the oxygen affinity. If this is correct

then the haemocyanin oxygen affinity, as expressed by the P_{50} would not be much different for both the 15°C and the 9.5°C cold acclimated crayfish. However the P_{50}content and total oxygen content in the haemolymph are lower in the cold acclimated crayfish, which experience a reduced metabolic demand and a higher oxygen availability at the lower water temperature. This indicates that the carrying capacity experiences a greater change during acclimation to a lower temperature than the oxygen affinity.

In Experiment E, the carrying capacity and the P_{50} of 10 Torr are the lowest. This should not be a problem as the PCO_2 of 1.9 Torr, at which the low P_{50} and capacity were recorded, is lower than the control value of 2.6 Torr from settled crayfish in water (Chapter 5), or the low recovery value of 2.1 Torr in water (Sample 12, Chapter 5). This suggests that such a low PCO_2 is only likely to be encountered during a period of high ventilation, and an associated high rate of oxygen delivery to the gills.

McMahon and Burggren (1988), summarised the P_{50} for a range of Crustacea, and although P_{50} varied from 6 to 28 Torr, it was not possible to conclude that an air breathing habit resulted in a higher P_{50}. A settled *P. zealandicus* with a haemolymph PCO_2 of 2.6 Torr would have a P_{50} of 12 Torr.

(b) <u>The effect of pH and other ions on P_{50}</u>. Changes in PCO_2, and hence changes in pH, produce changes in P_{50}, and this is known as the Bohr effect, ϕ, which is $\Delta log P_{50}/\Delta pH$.

The Bohr effect for *P. zealandicus* between pH 7.56 and 7.77 was - 0.48 for crayfish acclimated to 15°C and - 0.51 for cold acclimated crayfish, and - 0.96 for cold acclimated crayfish between pH 7.77 and 7.96. For the crayfish *A. pallipes* ϕ = - 0.3 to - 0.67 (Morris *et al.*, 1986b, 1986c), for *Astacus leptodactylus* ϕ = - 0.2 (Angersbach and Decker, 1978), for *Pacifastacus leniusculus* ϕ = - 0.3 to - 0.6 (Rutledge, 1981), and for *Orconectes rusticus* ϕ = - 0.63 to - 0.68 (Wilkes and McMahon, 1982). For the land crab, *Birgus latro*, ϕ = - 0.6 (Morris, Greenaway and McMahon, 1988), and for the blue crab, *Callinectes sapidus, under exercise, the Bohr effect* ϕ = - *1.14* (Booth *et al.*, 1982).

The effect of [urate], [lactate], [Ca^{+2}] and [caffeine] have been investigated in *A. pallipes* (Morris *et al.*, 1985, 1986a, 1986c, 1987). The Bohr effect remained between - 0.3 and - 0.55 and increases in all the variables reduced P_{50}. The reduction in P_{50} caused by increased [lactate] and [Ca^{+2}] has also been observed in the lobster *Homarus gammarus* (Taylor and Whiteley, 1989), the land crab *Birgus latro* (Morris, Greenaway and McMahon, 1988), the terrestrial hermit crab *Coenobite clypeatus* (Morris and Bridges, 1986), and the prawn *Palaemon elegans* (Bridges *et al.*, 1984). In the euryhaline crayfish *Pacifastacus leniusculus* (Wheatly and McMahon, 1982) and the intertidal prawn *Palaemon elegans* (Morris, Taylor and Bridges, 1988) the P_{50} decreased as the external ion concentration increased.

(3) Bicarbonate Buffering

P. zealandicus, when settled in water had a calculated [HCO_3^- + CO_3^{-2}] of 10 to 14 meq/l (from Chapter 5) which is similar to the control value of 11 to 14 meq/l recorded by Ball (1987). The experimental $\Delta CCO_2/\Delta pH$ was - 3.8 mmol/l/pH unit, and Ball (1987) measured a $\Delta CCO_2/\Delta pH$ of - 4.9 mmol/l/pH unit for *P. zealandicus*.

Changes in haemolymph PCO_2, brought about by changing from aquatic and aerial respiration, and subsequent changes in [HCO_3^-], [CO_3^{-2}] and [H^+], reflect the ability of crayfish to buffer haemolymph pH as the PCO_2 rises or falls. After 8 hours settling in water the P_aCO_2, pH_a and C_aCO_2 had returned to the initial values (see Figures 7.4 to 7.7).

The expected respiratory acidosis occurred during the first 12 hours in air, and the rise in C_aCO_2 associated with a rise in PCO_2 indicates a degree of metabolic compensation. There was a change in pH_a from 7.62 to 7.55, in P_aCO_2 from 2.6 to 6 Torr, and the C_aCO_2 had increased by 4 $mmol.l^{-1}$. After 24 hours in air this was almost fully compensated with a return to pH 7.65. The decrease in [H^+] represents a metabolic alkalosis which may in part be associated with the disappearance of the lactate which had returned to normal levels (Figure 5.8). There are pH oscillations of 0.1 units, approximately, from 12 to 24 to 36 to 48 hours in air. These

pH shifts, which are largely metabolic, are summarised in the Davenport diagrams Figures 7.4 and 7.6, and have been discussed in Chapter 5 as a diel rhythm.

Taylor and Wheatly (1981) report a minor secondary acidosis in *A. pallipes* after 24 hours in air. It is not possible to determine if the effect is similar to that observed in this experiment as there is no indication what time of day the measurements were taken.

A. pallipes was subjected to aerial respiration for 24 hours and demonstrated a sustained acidosis which was compensated for by an increase in $[HCO_3^-]$, and Taylor and Wheatly (1981) consider that the most likely source for both the concurrent increase in $[Ca^{+2}]$ and $[HCO_3^-]$ was exoskeleton $CaCO_3$. *Cancer productus* also experienced an increase in haemolymph $[Ca^{+2}]$ and CCO_2 when exposed to air, and an experimental PCO_2 which was lower than the calculated PCO_2 (deFur *et al.*, 1980), and the most likely source for both the Ca^{+2} and HCO_3^- ions was considered to be the exoskeleton $CaCO_3$.

The C_aCO_2 of 7 $mmol.l^{-1}$ in settled *A. pallipes* (Taylor and Wheatly, 1980; see Table 5.6) is lower than the 11 $mmol.l^{-1}$ in settled *P. zealandicus* (Table 7.3). Ball (1987) indicates that the haemolymph $[Ca^{+2}]$ in settled *P. zealandicus* is about 10 $mmol.l^{-1}$, which is similar to *A. pallipes* (Morris *et al.*, 1986b)

Aerial respiration by *P. zealandicus* produced no initial change in pH_a, a partially compensated acidosis in 12 hours, followed by pH oscillations which are largely of metabolic origin. The return to the water and aquatic respiration resulted in a rapid decline in the C_aCO_2 and P_aCO_2 and is a partially compensated respiratory alkalosis. The final settling period in the water between 8 am and 4 pm shows a metabolic alkalosis of 0.05 pH units, and may represent the aquatic equivalent of the pH oscillations observed when the crayfish were in air.

(4) Measured and predicted CCO_2 and PCO_2

There are discrepancies between the measured and calculated values for haemolymph CCO_2 and PCO_2 in the results from Chapter 5, summarised in Table 7.3, and the measurements

to determine the buffer capacity of crayfish haemolymph. This difference even persisted in the measurements from haemolymph equilibrated in a tonometer, and can be seen in Figure 7.6 as the difference between the measured CCO_2 and calculated CCO_2 lines, and is about 2 $mmol.l^{-1}$.

Because this difference appears in two different experiments the likelihood of a measuring error is considerably reduced. As the CCO_2 values reported here for settled *P. zealandicus* are similar to the values, between 10 and 14 $mmol.l^{-1}$, reported by Ball (1987) for control crayfish, it is unlikely that this value is in error. The haemolymph from the crayfish in the experiment in Chapter 5 may not have been in equilibrium, but this dis-equilibrium has persisted in the buffer-line determinations which used a tonometer.

An experimental PCO_2 lower than the calculated PCO_2 has also been reported for *Cancer productus* (deFur *et al.*, 1980). Involvement of exoskeleton $CaCO_3$ in an elevated haemolymph CCO_2 cannot be discounted, but will require measurement of $[Ca^{+2}]$, and without this the discrepancy will remain unsolved.

(5) Aerial Respiration

P. zealandicus appears to control the haemolymph pH, as can be seen in both the experimental (Figure 7.4), and calculated (Figure 7.6), Davenport diagrams. The pH-PCO_2-CCO_2 changes after emersion are dominated by a large increase in CCO_2 indicating metabolic compensation for a respiratory acidosis and a diel pH oscillation.

A change of 0.1 pH unit has been observed in *A. leptodactylus* in the evening prior to nocturnal activity (Massabuau *et al.*, 1984; Sakakibara *et al.*, 1987), and appears to be driven by an increase in ventilatory requirement resulting in a more alkaline haemolymph, with associated Bohr shift and increase in oxygen affinity. That *P. zealandicus* and *P. planifrons* are nocturnally active has been adequately demonstrated by Quilter (1975) and Devcich (1979). The pH shift has not been studied in undisturbed animals in water, and consequently the discovery of an apparent diel pH shift, observable after the crayfish had moved into the air, was a surprise.

As mentioned previously, increases in other species in both [Ca^{+2}] and [lactate] help to improve oxygen uptake by reducing P_{50}. The observations during aerial respiration (Chapter 5) indicate that as [lactate] starts to fall the CCO_2 is increasing. The source for the increase in CCO_2 has been attributed to carapace $CaCO_3$ and been associated with substantial [Ca^{+2}] increases in the haemolymph (deFur *et al.*, 1980; Taylor and Wheatly, 1981; Morris *et al.*, 1986b). An increase in haemolymph [Ca^{+2}] associated with the increase in CCO_2 could assist *P. zealandicus* during aerial respiration by increasing oxygen affinity.

It is clear that *P. zealandicus* is able to compensate for the acidosis which an aquatic creature experiences upon using aerial respiration. The crayfish demonstrates a remarkable control of haemolymph pH while using aerial respiration, not only maintaining pH, but apparently oscillating it to meet changing daily metabolic cycles. This would enable the animal to use aerial respiration to migrate to other water bodies across land.

Conclusions.

The crayfish acclimated at 9.5°C had similar P_{50} to the crayfish kept at 15°C, and a reduced oxygen capacity.

At 15°C a settled *P. zealandicus* with a haemolymph PCO_2 of 2.6 Torr would have a P_{50} of 12 Torr.

The Bohr effect was - 0.48 to - 0.51.

The haemolymph changes after emersion are dominated by increases in PCO_2 and CCO_2 indicating metabolic compensation for a respiratory acidosis.

Crayfish in air demonstrated a diel pH oscillation in haemolymph pH which was 0.1 units more alkaline in the evening than the morning. It is speculated that this produced a Bohr shift and an associated increase in oxygen affinity.

It is suggested that, as in other Crustacea, the source of the additional [HCO_3^-] when the crayfish were using aerial respiration may be from exoskeleton $CaCO_3$, which would result in a concurrent rise in [Ca^{+2}], and assist the crayfish by increasing haemolymph oxygen affinity.

CHAPTER 8

GAS EXCHANGE, VENTILATION AND PERFUSION IN WATER AND IN AIR

I. INTRODUCTION

Crayfish have been reported leaving the water in the search for food (Huxley, 1896) and to colonise new bodies of water (Williams and Hynes, 1976), and it was demonstrated in Chapter 3 that *P. zealandicus* also emerges from the water voluntarily. In air the crayfish must function without the hydraulic support and buoyancy provided by the aquatic environment. The lack of support and buoyancy will be felt most by soft tissues like the gills and through arthrodial membranes. The cylindrical filaments on a crayfish gill would not be as sensitive to collapse or distension as the flat blood spaces in the lamellae of a crab gill (Taylor, 1990), but the loss of internal pressure may still cause collapse of gill filaments or the gill stem.

Air provides less resistance to the scaphognathite, and Taylor and Wheatly (1980) point out that the scaphognathite is more effective as a water pump than an air pump; however this is offset by the increase in the oxygen content in air. The difficulty of maintaining ventilation and circulation during aerial respiration needs to be overcome if the animal is to be able to obtain sufficient oxygen in air.

The difference between haemolymph C_aO_2 and C_vO_2 is a measure of the quantity of oxygen taken up at the gills and is the quantity which the animal has used at the tissues. The amount of oxygen which the haemolymph can carry needs to be sufficient for the crayfish to maintain a settled $\dot{M}O_2$ in air, and preferably, to be able to engage in some activity. When the supply of oxygen is not sufficient the ventilation system, which supplies oxygen to the respiratory surfaces, and the circulation system, which removes oxygen in the haemolymph, are adjusted in an attempt to increase the supply.

The aim in this chapter is to measure the P_aO_2 and P_vO_2 of the crayfish while it is in the air and compare this with the P_aO_2 and P_vO_2 of the crayfish in the water. The frequency of the scaphognathite, f_R, and the heart, f_H, will be measured from crayfish while they are settling in water, exposed to aerial respiration for 48 hours, and then recovering in water. This data will be used in conjunction with the oxygen equilibrium curves to determine the oxygen content of the blood and how much is being delivered to the tissues.

II. MATERIALS AND METHODS

(1) Collection of animals

Crayfish of both sexes were collected from a small stream flowing into Lake Georgina, and ovigerous females and animals with missing chelae were returned to the stream. The animals were kept in the Zoology Department aquarium room in 70 cm x 40 cm x 50 cm deep tanks supplied with bore water and surplus refuges made from plastic tubing. The aquarium room had a 12 hour day 12 hour night light cycle and was kept at 15 ± 1°C. The crayfish were fed *ad lib.* and were judged to be at the intermoult stage (stage C) of the moult cycle.

(2) Pre and Post Branchial PO_2

There are problems associated with taking consecutive pre and post branchial haemolymph samples from the same crayfish:

a) removal of the first sample causes a drop in fluid pressure with the result that any sample taken immediately subsequent to this is a disturbed sample,

b) the animal does not have sufficient haemolymph to easily accommodate the removal of two successive samples.

Consequently a protocol was used which involved the random sampling of crayfish. Lengths of plastic tubing, of similar dimensions to the respirometers used for measuring aquatic and aerial oxygen consumption, were numbered 1 to 36 and put into a box. The crayfish were weighed, sexed and put into a piece of plastic tubing randomly selected from the box. Each end of the tube was covered with a coarse 1 mm

open weave nylon mesh which was secured with a tight fitting plastic ring (Figure 5.1). The tubes were laid out, and kept submerged, on a perforated tray supported on several bricks in a large tank (Figure 5.2) supplied with a continuous flow of bore water.

For the aquatic measurements the crayfish were settled in the water for 24 hours and for the aerial measurements the crayfish were settled in the water for 8 hours and then the water was lowered till it was below the perforated tray. The tank was covered with a sheet of expanded styrene foam to prevent fluctuations in air temperature and ensure that the airstones were able to maintain the humidity at saturation when the crayfish were out of the water.

Crayfish pre and post branchial haemolymph samples were taken after crayfish had been:

a) settled in water for 24 hours, and

b) settled in water for 8 hours then in air for 24 hours.

Crayfish were randomly removed from the tank. If the tube had an odd number, a postbranchial sample was removed from the pericardial sinus of the crayfish, and if the tube had an even number, a prebranchial haemolymph sample was taken through the arthrodial membrane from a sinus at the base of the cheliped. When a sample of haemolymph could not be obtained within 20 seconds the animal was released into a recovery tank.

Each haemolymph sample needed to be a minimum of 0.5 ml, and experience indicated that PO_2 and pH could be measured without clotting if the sample was kept on ice and processed promptly. It was possible to process eight animals in an hour. Some 36 animals could be easily accommodated in the experimental tank (Figure 5.2) so it was possible to do the experiment with 18 samples of both pre and post branchial haemolymph.

The haemolymph pH and PO_2 were measured with an AVL blood-gas analyzer. The pH electrode was calibrated with Radiometer precision buffer solutions, S1500 and S1510, which read 6.900 and 7.445 respectively at 15°C. The barometric

pressure was read twice each day and the PO_2 electrode was calibrated with a low flow of water saturated air at ambient temperature, which produces condensation when reduced to 15°C.

One trial was conducted with 36 crayfish settled in the water for 24 hours, and two trials with a total 67 crayfish settled in the air for 24 hours.

(3) f_H and f_R and P_{Branch}

A fine hypodermic needle was used to make a pair of small holes in the carapace on either side of each scaphognathite. The shadow of scaphognathite movement can be seen in good light and the holes were dorsal and ventral to this. Fine holes were also made on either side of the heart near to the branchiocardiac groove. Silver wire electrodes, bent into a 0.5 - 1.0 mm hook, were inserted through the holes and the leads were strapped down with strips of rubber dam and cyano-acrylate glue. The leads from the two scaphognathites and the heart were then brought together and secured to the dorsal carapace with cyano-acrylate and rubber dam strips.

Any two of the three electrode pairs could be plugged into a two channel Bioscience A100 Impedance Coupler, and the impedance signal was recorded on a BBC SE120 two channel flatbed recorder.

The experimental conditions were:-

Settling - started between 10.00 am and 11.00 am. Heart and scaphognathite recordings were taken at the beginning, and after 3 and 8 hours, samples 1, 2, and 3. The final recordings of settling in water were taken between 6.00 pm and 7.00 pm.

Emersion - achieved by lowering the water level. Heart and scaphognathite recordings were taken at the beginning, and after 1, 3, 12, 24, 36 and 48 hours, samples 4 to 10. Emersion was started as close to 8.00 pm as possible to ensure that samples 4, 7, 8, 9 and 10 were taken as near to 8.00 am and 8.00 pm as possible.

Recovery - achieved by raising the water level and resubmerging the crayfish. Recovery was monitored, and heart and scaphognathite recordings were made at the

beginning and after 1, 3 and 10 hours, samples 11, 12, 13 and 14.

Controls – a group of animals were settled in water between 3.00 and 4.00 pm, day 1, and heart and scaphognathite recordings were made at the beginning and between 8.00 and 9.00 pm., and 11.00 pm and midnight. On days 2 and 3 recordings were made between 8.00 and 9.00 am., 8.00 and 9.00 pm., 11.00 pm and midnight, on day 4 between 8.00 and 9.00 am., and 8.00 and 9.00 pm., and on day 5 the final recordings were taken between 8.00 and 9.00 am.

Changes in branchial chamber pressure, caused by the beating of the scaphognathite, were also measured. Impedance electrodes were placed on either side of the scaphognathite. Branchial chamber pressure was measured through a cannula which passed through the branchiostegite. The surface of the branchiostegite was sanded with a 20 mm diameter emery disk, to remove spines and lumps. With a dental burr a 2 mm diameter hole was carefully made through the centre of the branchiostegite and into the branchial cavity. A 1.5 mm diameter hole was drilled down the centre of a length of 8 mm perspex rod which was then cut into 5 mm disks. The flat faces of the disks were sanded smooth with 280 grain and 600 grain wet and dry sand-paper. Portex polythene tubing (ID 1.14 mm, OD 1.57 mm) was then pushed into the 1.5 mm hole, through the disk, and the end cut off at 45° with no more than 2 mm protruding. The disk was then attached to the carapace with cyano-acrylate glue with the tip of the cannula inserted into the hole in the branchiostegite. At the end of the experiment the disk with cannula were easily removed by inserting a sharp edge between the disk and the cuticle.

The impedance electrodes by the scaphognathite were connected to a two channel Bioscience A100 Impedance Coupler, and the branchial chamber pressure was recorded by a Bell and Howell 4-327 pressure transducer which was amplified by a Gould 13-4615-58 Universal amplifier. The impedance and pressure signals were recorded on a BBC SE120 two channel flatbed recorder.

(4) Data analysis and statistical methods

The haemolymph P_aO_2, P_vO_2, pH_a and pH_v are presented as the mean ± 1 standard deviation, and ± 1 standard error. Like the other variables the statistics for the pH are calculated directly and not as $[H^+]$ (Boutilier and Shelton, 1980). Differences were tested with an unpaired two tailed "t" test.

The f_R and f_H were analyzed with oneway Anova and differences between the samples were identified with the Tukey-Kramer Multiple Comparisons Test.

Linear regression was carried out between branchial pressure and the scaphognathite frequency and the branchial (diastolic - systolic) pressure and scaphognathite frequency.

The heart and scaphognathite frequencies from crayfish out of the water and from control crayfish settled in water were compared between morning and evening (8 am and 8 pm) samples with paired two tailed "t" tests. The morning samples 6 and 8 were compared with evening samples 7 and 9 from the crayfish in air, and morning samples 18, 21, 24 and 26 were compared with evening samples 16, 19, 22 and 25 from the control crayfish.

The data was analyzed with the InStat version 2.04 computer package from GraphPad Software. Results are tested at the 5% level of significance and the data are presented in tables as the mean ± 1 standard deviation, and ± 1 standard error, and graphically as the mean ± 1 standard error (SEM).

III RESULTS

(1) Pre and Post Branchial PO_2

Arterial and venous PO_2 and pH are presented in Table 8.1. Using data from the oxygen equilibrium curve and the Bohr shift, the P_{50} was calculated for the arterial and venous oxygen in both the aerial and aquatic trials, and an oxygen equilibrium curve with the PO_2 values and the oxygen contents is presented in Figure 8.1.

Calculations from the data in Table 7.1 provided a P_{50} of 16.0 Torr at pH 7.63, a P_{50} of 15.5 Torr at pH 7.66, and a P_{50} of 15.3 Torr at pH 7.67.

Table 8.1 The pre and post branchial oxygen tension and pH from the haemolymph of crayfish which had been settled in water for 24 hours, and crayfish which had settled in water for 8 hours and then in air for 24 hours.

		Pre-branchial samples			Post-branchial samples		
		Weight g	pH	PO_2 Torr	Weight g	pH	PO_2 Torr
Settled in water for 24 hours	n	18	18	18	18	17	18
	mean	24.06	7.67	17.21	22.73	7.63	48.68
	± stdev	6.13	0.08	9.93	3.72	0.10	27.65
	± sem	1.45	0.02	2.34	0.88	0.03	6.52
Settled in water for 8 hours, in air for 24 hours	n	34	31	30	33	31	31
	mean	31.11	7.66	8.39	28.11	7.67	11.42
	± stdev	8.44	0.08	2.07	7.60	0.07	5.03
	± sem	1.45	0.02	0.38	1.32	0.01	0.90

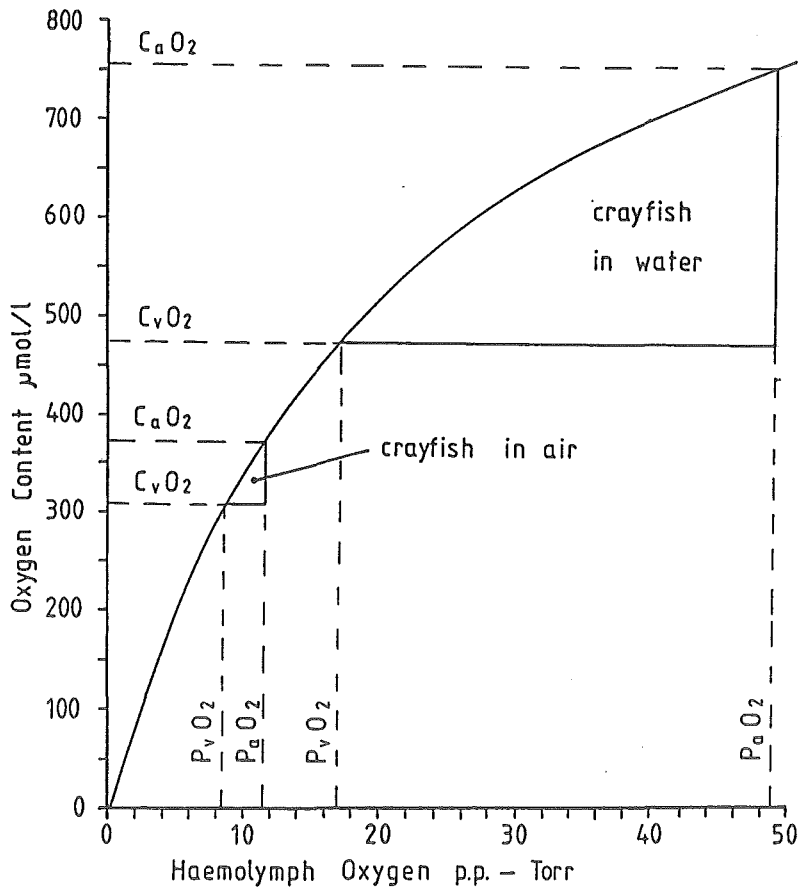


Figure 8.1 An oxygen equilibrium curve with the values from Table 8.1, indicating the oxygen content in the crayfish haemolymph at the arterial and venous PO_2 of crayfish settled in water for 24 hours, and crayfish settled in water for 8 hours and then in the air for 24 hours. The oxygen equilibrium curve was drawn from the data in Chapter 7. This curve assumes that the pre and post branchial pH are the same, see text for pre and post branchial pH comparisons.

There was an extremely significant difference between pre and post branchial PO_2 from the crayfish settled in the water (t = 4.545, 34 df., P < 0.0001) and a very significant difference between pre and post branchial PO_2 from the crayfish which had been subjected to aerial respiration for 24 hours (t = 3.050, 59 df., P = 0.0034). Comparing pre and post branchial groups in each treatment, there was no difference in haemolymph pH in the crayfish settled in water (t = 1.133, 33 df., P = 0.2654) or in the crayfish used for aerial respiration (t = 0.4801, 60 df., P = 0.6329), and there was no difference in the weight of crayfish settled in water (t = 0.7857, 34 df., P = 0.4375) or crayfish used for aerial respiration (t = 1.529, 65 df., P = 0.1311).

Using the equation from Taylor and Taylor (1992):

$$L_{diff} = (P_mO_2 - P_aO_2) / (P_mO_2 - P_vO_2)$$

where;

P_mO_2 = average ambient PO_2 at exchange surface,

P_aO_2 = post branchial PO_2,

P_vO_2 = pre branchial PO_2.

L_{diff} was estimated using a P_mO_2 between 140 and 150 Torr at the gas exchange surface.

In water L_{diff} was between 0.74 and 0.77, and in air L_{diff} was between 0.97 and 0.98.

(2) f_H and f_R and P_{Branch}

Recordings of the scaphognathite frequency and branchial pressure were made from six crayfish as they settled in water over a period of twenty four hours.

The regression of branchial pressure (mm water pressure, negative to ambient pressure) and scaphognathite frequency (between 40 and 120 bpm, Figure 8.2 Top) produced the equation:

Pressure = 0.3747 × frequency - 8.068

The regression of the difference between diastolic and systolic pressure in mm water pressure and scaphognathite frequency (Figure 8.2 Bottom) produced the equation:

Pressure = 2.451 - 0.002119 × frequency, and the slope was not significantly different from zero.

There was no evidence of flow reversal in the recordings of f_R and branchial pressure. *P. zealandicus* had an increase in mean branchial pressure from -1.8 mm to -4.8 mm water pressure between 40 and 120 bpm.

The heart and scaphognathite recordings of the crayfish were examined when they were first returned to the water after the electrodes were attached, after emersion, and after recovery was initiated.

The f_H was regular when the crayfish were first returned to the water after the electrodes had been attached. When the crayfish were first subjected to aerial respiration there was an increase in the size of the impedance signal, but no change in the f_H which remained steady, and as the crayfish settled in air the size of the impedance signal reduced. Recovery in water was initiated by immersion and the heart produced an increase in the size of the impedance signal, which decreased as recovery progressed, and the f_H remained steady.

The scaphognathites produced a very irregular beat, both in frequency and the amplitude of the impedance recording, when the crayfish were first returned to the water. This settled quickly. When the crayfish emerged the response was very variable. In some animals both scaphognathites produced a very irregular beat, in others one scaphognathite maintained a strong regular beat, while the other was very erratic or produced a very weak impedance signal. The crayfish settled rapidly in air and usually maintained a steady beat by the time three hours had elapsed. When the crayfish were again returned to the water the scaphognathite rates were similar to emersion. The irregular beats were again maintained for several hours and the animals had settled after 10 hours back in the water. In the settled crayfish f_H and f_R were continuous and regular.

It was observed that one scaphognathite had a higher frequency beat than the other, particularly when the animal was under stress, or when there appeared to be an increase in ventilatory need. Therefore the distinction was not made

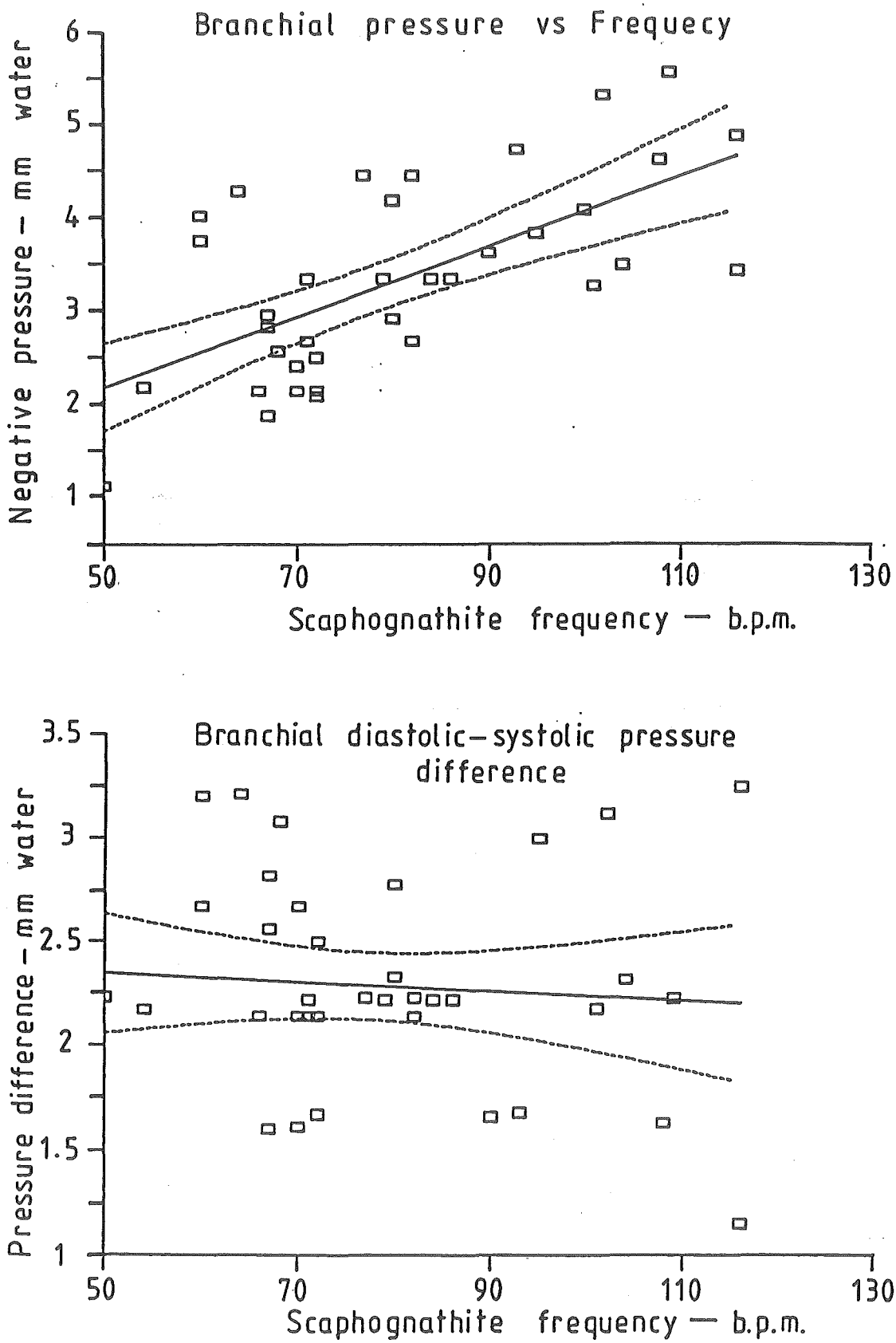


Figure 8.2 **Top**, the branchial pressure of P. zealandicus in mm water pressure (negative) plotted against the scaphognathite frequency, and **Bottom**, the difference between diastolic and systolic pressure plotted against the scaphognathite frequency.

between the left and right scaphognathites, but between the faster scaphognathite with the higher beat frequency and the slower scaphognathite with the lower frequency. The difference in scaphognathite frequency was more difficult to determine in the control animals as they were not disturbed by the process of emersion but remained in the water for 89 hours and were consequently more settled. In the 21 animals used in the experimental and control groups, 11 crayfish had a fast right and 10 a fast left scaphognathite.

Recordings of the scaphognathite and heart frequencies were made from 21 animals. There were 9 animals weighing 37.5 ± 12.7 g (± 1 SD) in the control group, and 12 animals weighing 39.3 ± 9.2 g (± 1 SD) in the experimental group. The temperature was 15.2 ± 0.4 °C.

The Anova of the heart rate produced a significant result, $F_{(25,202)} = 2.295$, $P = 0.0008$. The data are summarised in Table 8.2. The heart beat frequencies of the experimental animals are plotted in Figure 8.3 and the control animals are plotted in Figure 8.4.

The Anova of the fast scaphognathite produced a significant result, $F_{(25,186)} = 5.370$, $P < 0.0001$. The data are summarised in Table 8.3. The scaphognathite frequencies of the experimental animals are plotted in Figure 8.5 and the control animals are plotted in Figure 8.6.

The Anova of the slow scaphognathite produced a significant result, $F_{(25,189)} = 3.681$, $P < 0.0001$. The data are summarised in Table 8.4. The scaphognathite frequencies of the experimental animals are plotted in Figure 8.7 and the control animals are plotted in Figure 8.8.

During the period in air f_H was similar to that in water. The scaphognathites produced a different pattern, with both the fast and slow scaphognathite declining in frequency as the period in air become longer. There was no evidence of ventilatory pauses of one or both scaphognathites.

Comparison of morning and evening scaphognathite and heart rates produced significant differences in the control crayfish settled in water, and no significant differences in the crayfish exposed to aerial respiration.

Table 8.2 Heart beat frequency of experimental animals, settling in water, 48 hours in air, and recovering in water, and control animals. The column "p < 0.05" lists the significant pairwise comparisons between samples.

Summary of results measuring the heart rate

				Heart rate			
				Mean	S D	SEM	
	#	hour	n	--- beats per minute ---			p < 0.05
Settling	1	0	12	71.75	22.86	6.60	ns
in	2	3	12	70.67	14.00	4.04	ns
water	3	8	12	64.50	18.59	5.37	ns
	4	10	12	62.42	18.88	5.45	ns
Breathing	5	11	7	59.00	18.75	7.09	ns
air	6	13	12	64.25	14.17	4.09	ns
for	7	22	11	63.36	11.65	3.51	ns
48	8	34	11	67.45	14.07	4.24	ns
hours	9	46	11	62.27	8.24	2.48	ns
	10	58	11	64.00	6.10	1.84	ns
Recovery	11	60	11	71.91	8.98	2.71	ns
in	12	61	4	70.00	9.70	4.85	ns
water	13	63	11	77.18	13.04	3.93	18,21,22
	14	72	11	63.36	14.25	4.30	ns
	15	0	3	85.00	22.00	12.70	ns
Control	16	5	9	61.67	20.60	6.87	ns
group	17	8	6	74.50	15.93	6.51	ns
	18	17	9	50.22	14.70	4.90	13
Settled	19	29	9	59.44	21.58	7.19	ns
in	20	32	6	68.33	17.85	7.29	ns
water	21	41	9	49.56	18.27	6.09	13
for	22	53	8	49.25	15.98	5.65	13
89	23	56	6	51.17	18.12	7.40	ns
hours	24	65	9	52.00	15.78	5.26	ns
	25	77	3	68.33	25.77	14.88	ns
	26	89	3	54.00	12.12	7.00	ns

The morning and evening heart frequencies of control crayfish were significantly different (t = 2.333, 28 df., P = 0.0271). The morning and evening frequencies of the fast scaphognathite of control crayfish were significantly different (t = 2.486, 21 df., P = 0.0241), and the difference in the slow scaphognathite was very significant (t = 2.480, 24 df., P = 0.0090).

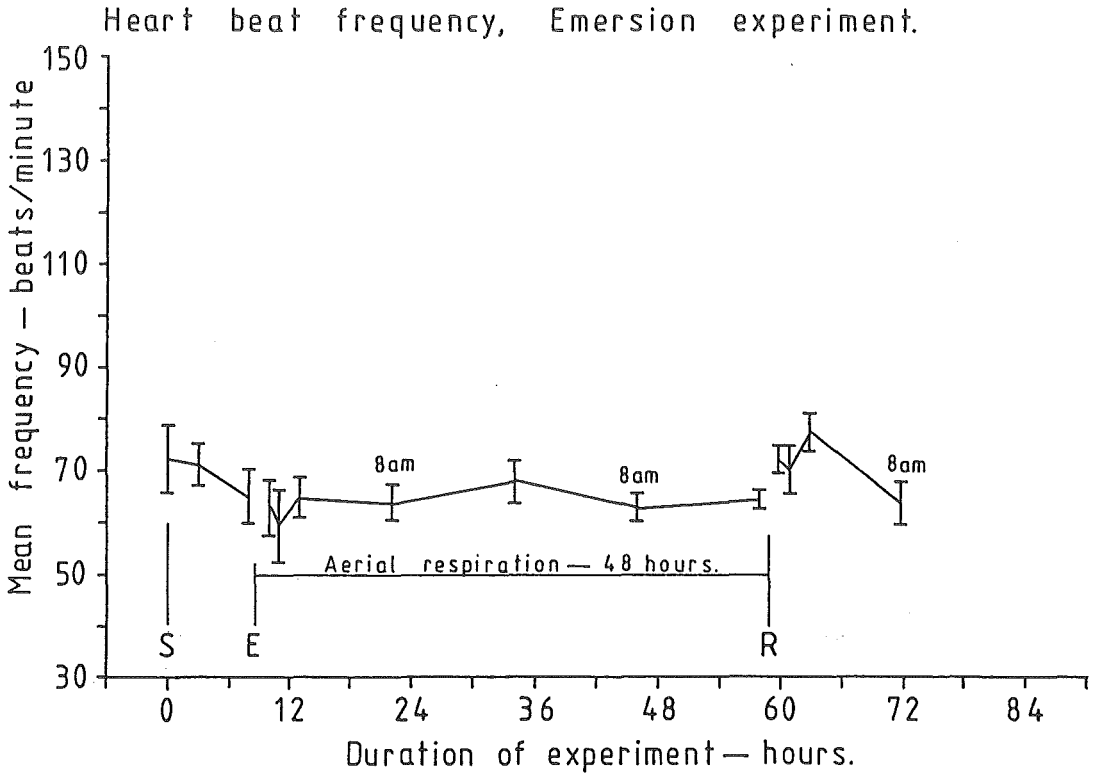


Figure 8.3 Plot of the heart rate of experimental crayfish settling in water, 48 hours in air and recovering in water. Data are the mean ± 1 sem, and the n values are in Table 8.2.

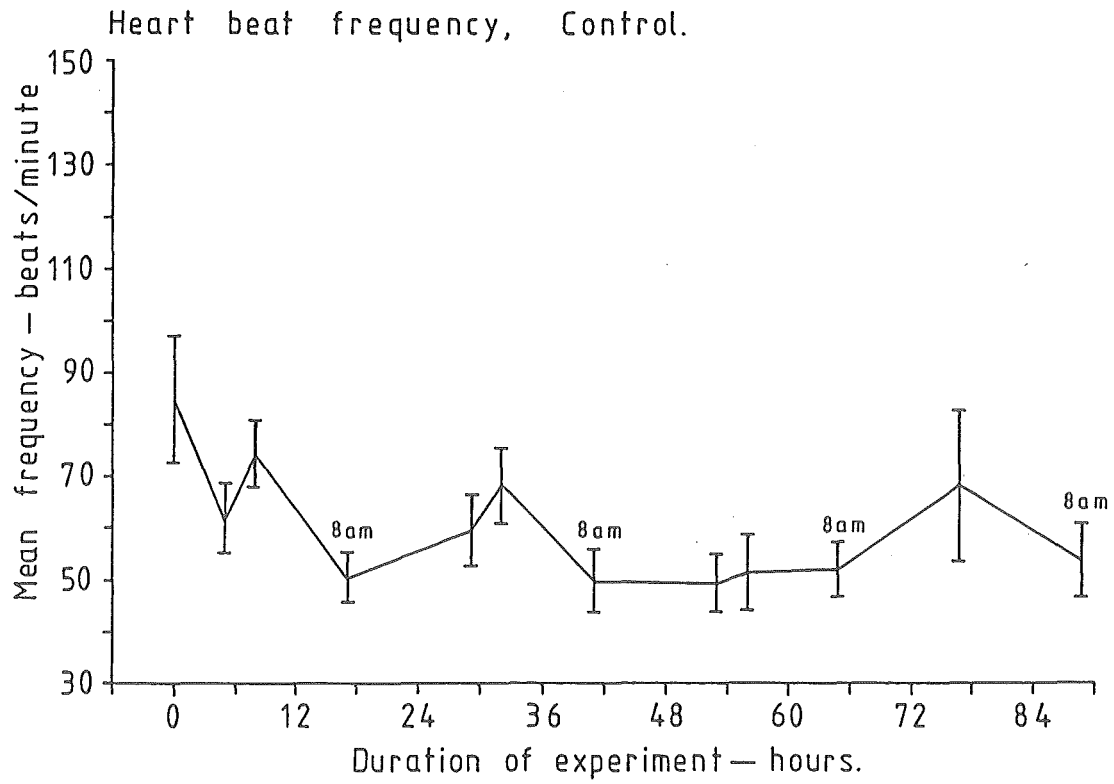


Figure 8.4 Plot of the heart rate of the control crayfish settling in water for 89 hours. Data are the mean ± 1 sem, and the n values are in Table 8.2.

Table 8.3 The fast scaphognathite of experimental animals, settling in water, 48 hours in air, and recovering in water, and control animals. The column "p < 0.05" lists the significant pairwise comparisons between samples.

Summary of results from the faster scaphognathite

				Faster scaphognathite			
				Mean	S D	SEM	
	#	hour	n	--- beats per minute ---			p < 0.05
Settling	1	0	12	122.25	33.17	9.58	11,14,18,19,21,22,23,24
in	2	3	12	112.00	41.27	11.91	14,18,19,21,22,24
water	3	8	12	110.75	41.90	12.10	14,18,19,21,22,24
	4	10	11	118.00	38.08	11.48	18,19,21,22,24
Breathing	5	11	7	118.00	37.85	14.31	18,19,21,22,24
air	6	13	12	118.08	44.08	12.72	14,18,19,21,22,23,24
for	7	22	10	85.50	29.60	9.36	ns
48	8	34	11	103.73	43.00	12.97	ns
hours	9	46	11	80.09	46.62	14.06	ns
	10	58	11	101.00	28.74	8.67	ns
Recovery	11	60	11	70.82	19.17	5.78	1
in	12	61	4	80.00	17.01	8.50	ns
water	13	63	11	78.82	43.45	13.10	ns
	14	72	9	58.00	8.50	2.83	1,2,4,6
	15	0	2	87.00	52.33	37.00	ns
Control	16	5	5	63.20	13.59	6.08	ns
group	17	8	4	97.50	29.14	14.57	ns
	18	17	8	50.75	10.17	3.59	1,2,3,4,5,6
Settled	19	29	8	54.50	9.34	3.30	1,2,3,4,5,6
in	20	32	4	68.25	30.41	15.21	ns
water	21	41	9	51.67	11.18	3.73	1,2,3,4,5,6
for	22	53	9	56.56	9.32	3.11	1,2,3,4,5,6
89	23	56	6	57.50	23.16	9.45	1,6
hours	24	65	7	51.14	9.97	3.77	1,2,3,4,5,6
	25	77	3	56.33	9.02	5.21	ns
	26	89	3	59.33	9.29	5.36	ns

The heart (t = 1.010, 21 df., P = 0.3239), fast scaphognathite (t = 1.665, 20 df., P = 0.1116) and slow scaphognathite (t = 0.9887, 21 df., P = 0.3341) frequencies of the experimental crayfish did not show a significant difference between morning and evening.

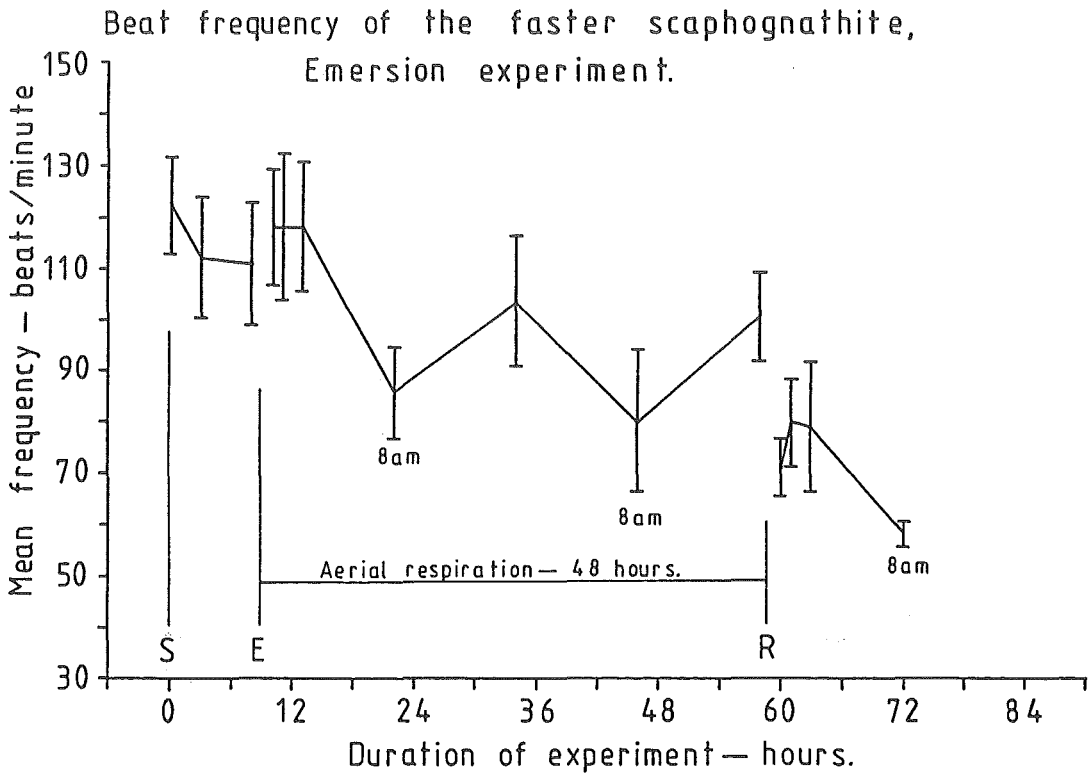


Figure 8.5 Plot of the beat frequency of the faster scaphognathite of experimental crayfish settling in water, 48 hours in air and recovering in water. Data are the mean ± 1 sem, and the n values are in Table 8.3.

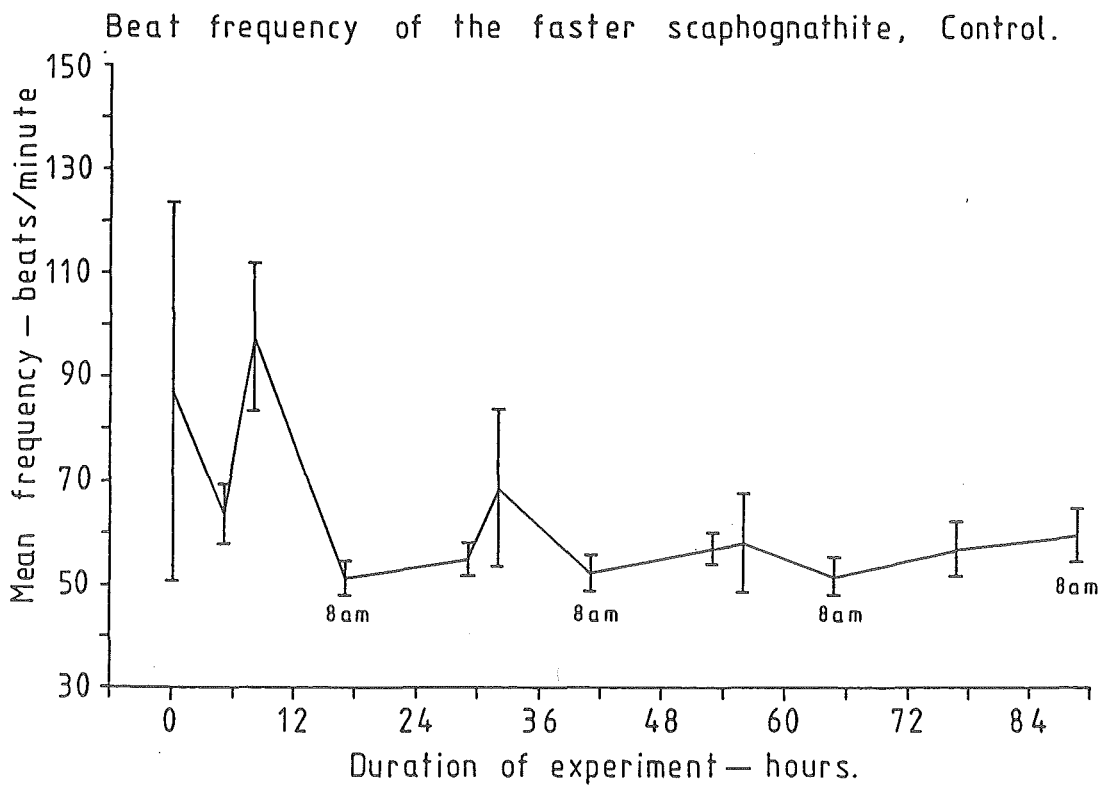


Figure 8.6 Plot of the beat frequency of the faster scaphognathite of the control crayfish settling in water for 89 hours. Data are the mean ± 1 sem, and the n values are in Table 8.3.

Table 8.4 The slow scaphognathite of experimental animals, settling in water, 48 hours in air, and recovering in water, and control animals. The column "p < 0.05" lists the significant pairwise comparisons between samples.

Summary of results from the slower scaphognathite

				Slower scaphognathite			
				Mean	S D	SEM	
	#	hour	n	--- beats per minute ---			p < 0.05
Settling	1	0	12	100.17	43.33	12.51	21,22,24
in	2	3	11	88.00	38.25	11.53	ns
water	3	8	11	94.91	33.27	10.03	ns
	4	10	12	89.08	30.90	8.92	ns
Breathing	5	11	7	96.57	25.18	9.52	ns
air	6	13	12	107.17	41.19	11.89	14,18,19,21,22,24
for	7	22	11	80.73	31.15	9.39	ns
48	8	34	11	97.82	40.56	12.23	21
hours	9	46	11	80.18	45.25	13.64	ns
	10	58	11	84.64	20.78	6.26	ns
Recovery	11	60	11	71.64	15.57	4.69	ns
in	12	61	4	81.25	18.46	9.23	ns
water	13	63	10	69.20	14.76	4.67	ns
	14	72	9	56.67	10.01	3.34	6
	15	0	2	86.50	51.62	36.50	ns
Control	16	5	6	64.00	11.68	4.77	ns
group	17	8	5	87.20	25.05	11.20	ns
	18	17	9	54.56	14.53	4.84	6
Settled	19	29	9	54.00	12.05	4.02	6
in	20	32	5	62.60	22.97	10.27	ns
water	21	41	9	48.22	8.97	2.99	1,6,8
for	22	53	9	52.78	7.10	2.37	1,6
89	23	56	5	56.00	18.55	8.29	ns
hours	24	65	7	46.86	9.79	3.70	1,6
	25	77	3	54.33	10.12	5.84	ns
	26	89	3	52.67	20.01	11.55	ns

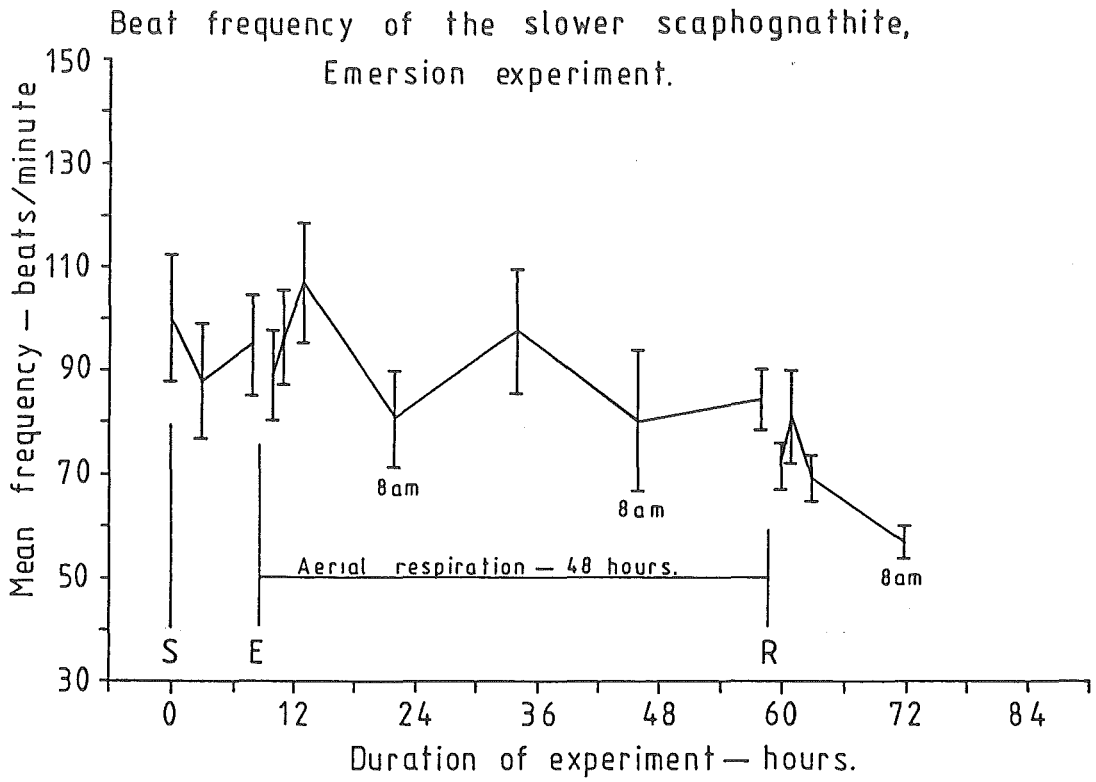


Figure 8.7 Plot of the beat frequency of the slower scaphognathite of experimental crayfish settling in water, 48 hours in air and recovering in water. Data are the mean ± 1 sem, and the n values are in Table 8.4.

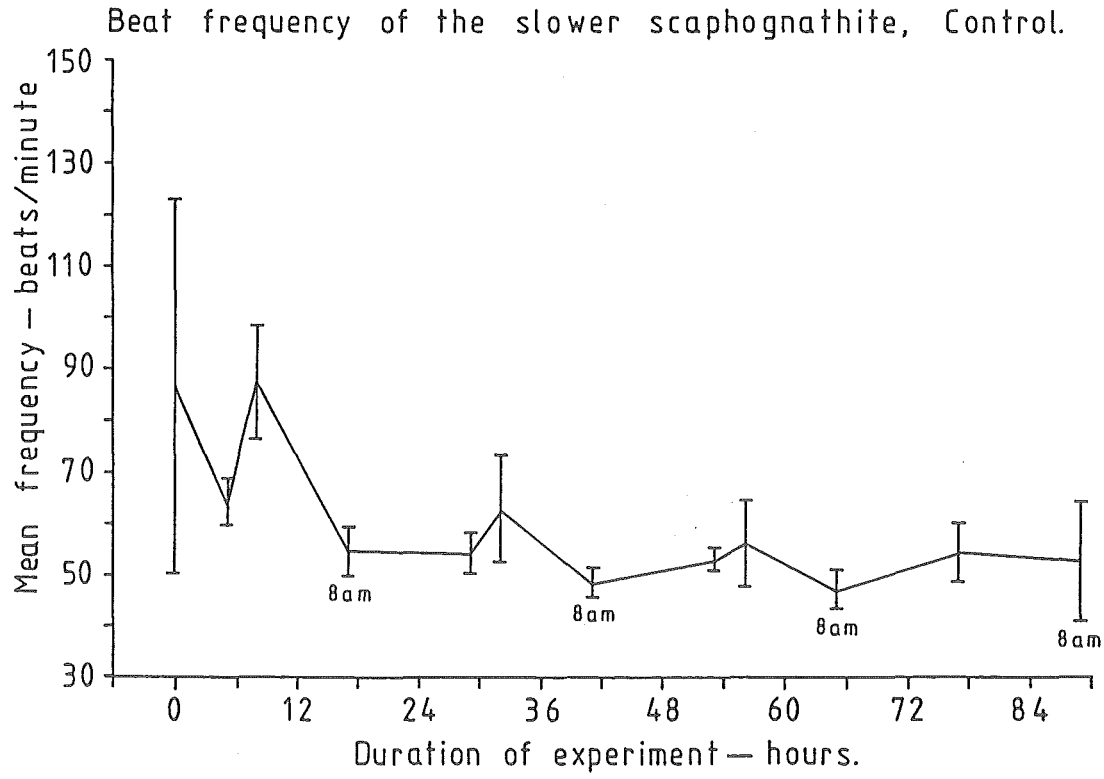


Figure 8.8 Plot of the beat frequency of the slower scaphognathite of the control crayfish settling in water for 89 hours. Data are the mean ± 1 sem, and the n values are in Table 8.4.

IV DISCUSSION

(1) Pre and Post Branchial PO_2

The quantity of oxygen taken up at the gills is measured by the difference between C_aO_2 and C_vO_2, and these are altered by pH, [lactate], [Ca^{+2}], [urate] and [caffeine] (Bridges *et al.*, 1984; Morris and Bridges, 1986; Morris *et al.*, 1985, 1986a, 1986c, 1987; Morris, Greenaway and McMahon, 1988; Taylor and Whiteley, 1989). These modulators alter the P_{50}, or oxygen affinity, of the haemolymph, and alter the quantity of oxygen which can be carried at any given PO_2. This has the greatest effect on oxygen content at the steep part of the oxygen equilibrium curve (Figures 7.2, 7.3 and 8.1) at low PO_2 values.

There was no difference in the pre and post branchial pH of the crayfish settled in water and settled in the air for 24 hours, and consequently no change in P_{50} or capacity between venous and arterial haemolymph through a change in pH at the gills. After 12 hours aerial respiration the crayfish haemolymph had experienced a [lactate] increase of 5 $mmol.l^{-1}$ (Chapter 5), which declined during the subsequent 12 hours.

The respiratory acidosis expected with aerial respiration occurred during the first 12 hours in air, and the associated rise in C_aCO_2 and PCO_2 indicate metabolic compensation. It was pointed out that in other Crustacea the increase in CCO_2 in excess of that expected from the increase in PCO_2 is from $CaCO_3$ sources in the carapace and is associated with an increase in haemolymph [Ca^{+2}].

Increases in both [lactate] and [Ca^{+2}] have been shown to produce a left shift to the oxygen equilibrium curve, and a reduction in P_{50}. McMahon (1988) points out that a left shift increases the slope of the curve considerably, enhancing the oxygen content of the haemolymph at low PO_2. Forgue *et al.* (1992) state that in settled crustaceans it is found that arterial PO_2 remains low, < 30 Torr, even when inspired PO_2 is as high as 300 Torr. A low P_aO_2 is sufficient to saturate a high affinity pigment which can be achieved with a lower ventilatory flow rate. Massabuau and Burtin (1984) reduced P_IO_2 from 250 Torr to 8 Torr and *Astacus leptodactylus* was

able to maintain $\dot{M}O_2$ down to 25 Torr, and maintained the haemolymph P_aO_2 and the water P_EO_2 within a narrow range independent of the P_IO_2.

The data and literature suggest that a crayfish which is settled in air may be able to maintain $\dot{M}O_2$ with a low P_aO_2, through a shift in the oxygen equilibrium curve produced by an increase in [lactate] followed by an increase in $[Ca_{+2}]$, which increase oxygen carrying capacity of the haemolymph.

L_{diff} is a measure of the diffusion/perfusion limitations on gas exchange at the gill. The change in the estimated L_{diff} of 0.7 from crayfish settled in water to the L_{diff} of 0.98 from crayfish which had been in air for 24 hours means that changes have occurred in oxygen uptake at the gills. Taylor and Taylor (1992) indicate that aquatic decapods with an L_{diff} between 0.5 and 0.8 in water are diffusion limited. The L_{diff} of 0.98 from crayfish in air indicates extreme limitation in diffusion, and is characteristic of an aquatic crayfish during aerial respiration (Taylor and Taylor, 1992). The $\dot{M}O_2$ of the crayfish in water and in air (Table 4.1, Figure 4.4) indicate that crayfish did not suffer a decline in oxygen uptake during 48 hours aerial respiration. The increase in L_{diff} means that if there is a decrease in diffusion then the perfusion rate must increase to maintain $\dot{M}O_2$.

(2) f_H and f_R and P_{Branch}

There was an increase in mean branchial pressure with increase in f_R, the branchial pressure changing from - 2 mm to - 4.5 mm water pressure with increase in f_R from 50 bpm to 120 bpm (Figure 8.2, Top). The diastolic-systolic pressure difference remained between 2.7 mm and 2.4 mm water pressure with an increase in f_R from 50 bpm to 120 bpm (Figure 8.2, Bottom). The relationship between the diastolic-systolic pressure difference and the scaphognathite frequency (Figure 8.2, Bottom) indicates that over the range of scaphognathite frequencies which were recorded there was no drop in efficiency. An f_R below 40 bpm was not recorded in this experiment.

Burggren and McMahon (1983) found that the f_R of *Orconectes virilis* settled in water remained constant, and

argue that reflux through the scaphognathite down the pressure gradient and back into the branchial chamber could render the pump ineffective below 40 bpm. They observed an increase in mean branchial pressure from -8 mm to -13 mm water pressure between 60 and 260 bpm.

During aerial respiration the f_H remained settled and demonstrated less fluctuations than f_H in control crayfish in water. In contrast, the f_R of the crayfish in air was more variable than the f_R in control crayfish. The crayfish did not experience an increase in f_H when emerging from the water and maintained a relatively stable f_H throughout the 48 hours in air, with the highest recorded f_H of 77 bpm after 3 hours recovery. Taylor and Wheatly (1981) found that during 24 hours aerial respiration the f_H of *A. pallipes* declined from 84 to 71 bpm. During the 48 hours in air *P. zealandicus* maintained a fluctuating, but not declining f_H between 59 bpm and 67 bpm.

In air *P. zealandicus* recorded the highest f_R during the first three hours, and for the next two days f_R oscillated, with a high value in the evening and a low in the morning. This effect potentially masked any elevation in the f_R which may have occurred when the crayfish was first transferred into air as the period of emerging coincided with the high f_R recorded in the evenings. Both reduced resistance to movement and reduced efficiency of the scaphognathite in air will affect changes in f_R resulting in larger changes in f_R in air than in water. In air the f_R and the f_H of *A. pallipes* were elevated for the first two hours, after which they recovered to submerged rates (Taylor and Wheatly, 1981). After 12 hours in air the f_H and f_R of *P. zealandicus* were not significantly different from the control values from crayfish settled in water.

In the previous chapters it was argued that during the recovery phase the crayfish had an elevated $\dot{M}O_2$ which quickly declined, and a P_aO_2 which remained elevated for at least 10 hours while the P_aCO_2 was very low, suggesting an elevated respiration to remove CO_2. However neither f_H or f_R are significantly elevated in the first hours of recovery

relative to the controls.

The f_H, and the f_R for both scaphognathites of the control crayfish exhibit a regular fluctuation, with high f_R and f_H recorded in the evening and low f_R and f_H in the morning. There is no significant difference between morning and evening f_R from crayfish in air, possibly caused by the reduced resistance to movement and reduced efficiency of the scaphognathite increasing the variability of the f_R.

Pollard and Larimer (1977) found a diel rhythm of f_H while the crayfish *Procambarus clarkii* was in the water. In Chapter 5 the rhythm of pH oscillations in P. zealandicus haemolymph were discussed. Daily changes in ventilatory requirement and pH have been observed in *A. leptodactylus* (Massabuau *et al.*, 1984; Sakakibara *et al.*, 1987).

The $C_aO_2 - C_vO_2$ difference of the crayfish settled in the water is about 4 fold the $C_aO_2 - C_vO_2$ difference of the crayfish settled in air (Figure 8.1). The $\dot{M}O_2$ of crayfish settled in air for 48 hours was not significantly different from crayfish settled in water (Chapter 4).

It was established in this chapter that there are no significant differences in f_H between crayfish settled in water and crayfish in air. It was mentioned, however, that there was an increase in the size of the impedance signal from the heart when the crayfish first emerged into air. This may indicate an increase in stroke volume, and an increase in perfusion. It has also been mentioned that increases in both [lactate] and [Ca^{+2}] have been shown to produce a left shift to the oxygen equilibrium curve, and a reduction in P_{50}, and this increases the slope of the curve considerably, enhancing the oxygen content of the haemolymph at low PO_2.

The difference in $C_aO_2 - C_vO_2$ between the crayfish settled in the water and the crayfish settled in air is likely to be a combination of:

- an increase in cardiac stroke volume, seen in the change in amplitude in impedance recordings,
- a change in the P_{50} possibly brought about by an increase in haemolymph [lactate] and [Ca^{+2}].

Conclusions

Crayfish in air for 24 hours demonstrated no difference in arterial and venous pH, and the possibility is discussed that an increase in [lactate] followed by [Ca^{+2}] may alter the P_{50} and increase the oxygen capacity at low PO_2.

Estimates of L_{diff} in water of 0.7, and in air of 0.98, indicate that respiration in *P. zealandicus* is diffusion limited.

The relationship between f_R and the diastolic systolic pressure difference indicates that the crayfish is able to maintain the efficiency of the scaphognathites between 40 bpm and 120 bpm.

During aerial respiration the f_H remained settled and demonstrated less fluctuations than f_H in control crayfish in water. In contrast, the f_R of the crayfish in air was more variable than the f_R in control crayfish.

The control crayfish settled in water exhibited a diel fluctuation in f_R and f_H, with high f_R and f_H recorded in the evening and low f_R and f_H in the morning.

CHAPTER 9

GENERAL DISCUSSION

Many of the Parastacoidea are described as semi-aquatic and even terrestrial, including species of *Cherax*, *Engaeus* and *Engaewa*, not because they are independent of water but because their burrows are often distant from water bodies. Some *Engaeus* are more highly modified for burrowing than the burrowers of any other group (Hobbs, 1988). Many species construct burrows which are not found in situations where they can be flooded and the crayfish within the burrows are active at all times of the year and never appear in open water (Horwitz and Richardson, 1986; Hogger, 1988). Some Australian crayfish have been found in artificial ponds miles from the nearest population (Hogger, 1988), suggesting they must be able to withstand the desiccation and physiological stresses involved in such a migration.

The New Zealand freshwater crayfish, *Paranephrops*, is also reputed to leave the water. The two species occur in lakes, ponds, streams and swamps, on gravel, rocky, vegetated or muddy substrates (Hopkins, 1970; Chapman and Lewis, 1976; Carpenter, 1977). They hide, probably from daytime predators (Scott and Duncan, 1967; Devcich, 1979), in dense aquatic vegetation, under large boulders and logs, or dig deep burrows where the substrate permits, especially if there is a strong current (Hopkins, 1970).

This study aimed to discover some of the mechanisms which *P. zealandicus* may employ to maintain respiration and sustain its metabolism while it is out of the water.

The gills of the Parastacoidea differ in number from the gills of the Astacoidea, and lack the epipodites found on the podobranchae of the Astacoidea (Hobbs, 1988; Holdich and Reeve, 1988). The gill formula, in *P. zealandicus*, of 20 gills + 1 rudimentary gill + 1 epipodite was confirmed. The crayfish does not have the epipodites found in the

Astacoidea, but does have a narrow membranous wing (Hopkins, 1970) which extends along the proximal half of the stem of the podobranch. It is possible that this may serve a structural function, especially when the crayfish is out of the water.

Dunel-Erb *et al.* (1982) attributed an ionic regulatory function to the epipodite-type laminae on the podobranchae of *Astacus*, which raised the question - where and how is this achieved in the Parastacidae, especially as there is evidence of very active mechanisms for regulating the ionic status of these freshwater crayfish (Wong and Freeman, 1976c; Greenaway and Lawson, 1982).

The transmission electron microscope micrographs and light micrographs provided evidence for two types of gill filament. The cellular differentiation indicated that the filaments are involved in two different functions, gas exchange, in the respiratory gill filament, and ion exchange, in the ion regulating filament (Fisher, 1972; Rogers, 1982; Dickson *et al.*, 1991). The respiratory filament has a cuticle which is 0.7 μm thick, an afferent and efferent vessel, and haemolymph lacunae adjacent to the cuticle. The ion regulating filament has a cuticle which is 1.2 μm thick, and the cells adjacent to the cuticle contain all the structures and membranes which have been associated with ion regulating gills and filaments in other Crustacea (Morse *et al.*, 1970; Felder *et al.*, 1986; Dickson *et al.*, 1991).

Crustacea which are bimodal breathers, or terrestrial, such as *Carcinus maenas* (Taylor and Butler, 1978), *Cardisoma guanhumi* and *Gecarcinus lateralis* (O'Mahoney and Full, 1984), *Holthuisana transversa* (Greenaway, Bonaventura and Taylor, 1983; Greenaway, Taylor and Bonaventura, 1983), *Heloecius cordiformis* (Maitland, 1990), *Cyclograpsus lavauxi* (Innes *et al.*, 1986), and *Austropotamobius pallipes* (Taylor and Wheatly, 1980), appear to be able to maintain their oxygen consumption in air at levels similar to oxygen consumption in water.

Resting oxygen consumption in air is important, for if the crayfish cannot maintain adequate oxygen consumption in

air then emersion can only be used for brief excursions and cannot provide a refuge from environmental pressures. It was found that *P. zealandicus* emerged from the water voluntarily, and a settled crayfish was able to maintain oxygen consumption in air between 1.0 and 1.07 $\mu mol.l^{-1}.g^{-1}$ for an extended period of 48 hours. This was similar to its oxygen consumption of 1.1 $\mu mol.l^{-1}.g^{-1}$ when it was settled in water.

During recovery from 48 hours emersion the elevated $\dot{M}O_2$ was of similar duration and magnitude to that observed when the animal was first settling in the respirometer. The total oxygen consumption during eight hours recovery was similar to the oxygen consumption during the eight hours settling. It was argued that similar processes were at work, and that the period of emersion produced no oxygen deficit other than a reduction in haemolymph oxygen content, seen as a drop in haemolymph PO_2.

Emersion and the subsequent loss of water from the branchial chamber may cause clumping of the gills reducing the area available for respiration, and increase resistance to gas diffusion with a water film increasing the effective thickness of the barrier between the air and the haemolymph. This is a common problem with bimodal crab and crayfish gills (Taylor and Wheatly, 1980; O'Mahony and Full, 1984; Innes *et al.*, 1986; deFur, 1988). An increase in resistance to gas diffusion would lead to a reduction in haemolymph PO_2 until the diffusion gradient had increased sufficiently to maintain the rate of diffusion needed to meet $\dot{M}O_2$. Although resting $\dot{M}O_2$ in air was the same as in water, a decline in haemolymph P_aO_2 from 90 Torr to 55 Torr indicated an increase in the diffusion gradient from the medium to the haemolymph. If a settled crayfish is able to maintain $\dot{M}O_2$ in air without an apparent deficit, it suggests that sufficient oxygen is able to pass from the air to the haemolymph and the gills do not limit aerial respiration of a crayfish at rest. The loss in haemolymph oxygen content is not recovered until conditions for gas exchange are again favourable.

There is an oxygen tension, P_{CRIT}, below which the oxygen consumption of an animal becomes compromised. At this point the oxygen availability is so reduced that the animal is no

longer able to maintain its resting $\dot{M}O_2$, even by increasing the effort put into respiration. This occurred at a P_IO_2 between 41 Torr and 45 Torr, and the oxygen consumption was 1.06 $\mu mol.l^{-1}.g^{-1}$. When the P_IO_2 fell below this the oxygen consumption declined rapidly.

Except for one measurement, the crayfish in air for 48 hours had a haemolymph P_aO_2 above P_{CRIT}, indicating that the crayfish were not experiencing internal hypoxia while in air and that oxygen availability was not compromised.

Elevated water temperature, increasing hypoxia in the water, and increasing the crayfish density by putting two animals in the experimental tank, did not stimulate a significant difference in voluntary emersion when compared with solo crayfish in oxygenated water at 18°C. The crayfish which experienced hypoxia down to 30 Torr did not display the emersion response observed in some crustaceans, using the oxygen in the air and removing the CO_2 in the water.

It is important for a bimodal animal to be able to move between water and air with minimal respiratory difficulty or compromise. With a settled aerial $\dot{M}O_2$ similar to its settled aquatic $\dot{M}O_2$, *P. zealandicus* is able to remain out of the water for up to 48 hours without a metabolic debt.

In water CO_2 is some 30 times more soluble than O_2, and in air oxygen makes up nearly 21% of volume. This is the source of a major difference between air breathing and water breathing animals. Taylor and Innes (1988) point out that the low availability of O_2 in water results in high ventilation rates in water-breathers. This is effective hyperventilation with respect to CO_2, which is so readily disposed of in water, and consequently aquatic animals typically have a haemolymph PCO_2 in the range of 2 to 4 Torr, and correspondingly low levels of CCO_2 (Taylor and Taylor, 1992). In air the haemolymph PCO_2 needs to be higher than the expired air PCO_2 if an air-breathing animal is to unload CO_2 at the respiratory surface, and in some vertebrate air breathers the venous PCO_2 may be as high as 40 Torr.

The changes in the haemolymph brought about by the move from aquatic respiration to aerial respiration, and back

again, were expected to reflect the difference in the disposal of CO_2 in air and water. The result would be an increase in haemolymph P_aCO_2 after the move to aerial respiration with an associated increase in $[H^+]$ producing a respiratory acidosis, and the reverse pattern would be observed when the crayfish returned to the water.

The change from aquatic to aerial respiration was accompanied by an increase in haemolymph PCO_2 and $[H^+]$. The expected respiratory acidosis occurred during the first 12 hours in air, with a change in pH from 7.62 to 7.55, but a large rise in C_aCO_2 associated with a rise in PCO_2 indicates a degree of metabolic compensation. The [lactate] had also increased to 5 $mmol.l^{-1}$. After 24 hours in air pH was almost fully compensated with a return to pH 7.65. The decrease in $[H^+]$ represents a metabolic alkalosis which may in part be associated with the disappearance of the lactate which had returned to normal levels. During the 48 hours in air there were oscillations of approximately 0.1 pH units, which are largely metabolic.

The return to the water and aquatic respiration resulted in a rise in pH from 7.65 to 7.71 in 3 hours and a rapid decline in the C_aCO_2 and P_aCO_2 and is a partially compensated respiratory alkalosis.

During the first 12 hours in air the crayfish experience a rise in haemolymph [lactate] which declines during the next 12 hours. This indicates that during the first 12 hours in air the level of activity exceeded the supply of aerobic energy, and that some time later this situation was reversed. The decline in haemolymph P_aO_2 during the period of aerial respiration indicated that there was an increase in the diffusion gradient from the medium to the haemolymph to maintain the $\dot{M}O_2$ in air, and it is speculated that during the first 12 hours in air the resistance to diffusion was at its highest. This is suggested by both the low P_aO_2 and the high [lactate] of 5 mmol/l.

The haemolymph changes during aerial respiration were dominated by the large increase in CCO_2 which compensated the expected respiratory acidosis. There was also a daily shift in pH. It is likely that as in other Crustacea the source of

the additional [HCO_3^-] was from the $CaCO_3$ in the exoskeleton. In other Crustacea this also resulted in a concurrent rise in haemolymph [Ca^{+2}].

The recovery in water after 48 hours aerial respiration was differenct from the settling period at the beginning of the experiment. During the recovery in water the crayfish maintained an elevated haemolymph P_aO_2 for 10 hours, which was kept high by the increased respiratory effort involved in the removal of the large quantities of haemolymph CO_2. This was indicated by the very rapid drop in P_aCO_2 which accompanied the return to water, and the rapid fall in C_aCO_2 to normal levels over the following 10 hours.

When the acidosis on emersion, alkalosis on submergence, and the pH oscillations while the crayfish is in the air are considered, it would seem that the crayfish are able to regulate pH_a during aerial respiration even when the ability to remove CO_2 is reduced.

Respiration is a process involving haemolymph oxygen carrying capacity, ventilation and circulation to move O_2 from the external environment to the tissues, and return the CO_2 produced to the external environment. One measure of the system is L_{diff}, which measures the diffusion/perfusion limitations on gas exchange at the gill. An estimate of L_{diff} for *P. zealandicus* in water is 0.7, and in air is 0.98, indicating that respiration is diffusion limited.

The relationship between f_R and the diastolic-systolic pressure difference indicates that the crayfish in water is able to maintain the efficiency of the scaphognathites between 40 bpm and 120 bpm. Taylor and Wheatly (1980) point out that the scaphognathite is more effective as a water pump than an air pump, and ventilation during aerial respiration needs to be maintained if the animal is to obtain sufficient oxygen. The lack of significant differences in f_R and f_H between crayfish in water and in air, coupled with the maintenance of $\dot{M}O_2$, indicates that perfusion was adequate. An increase in the size of the impedance signal from the heart was observed when the crayfish first emerged into air, and may indicate an increase in stroke volume, and perfusion.

At 15°C a settled *P. zealandicus* with a haemolymph PCO_2 of 2.6 Torr would have a P_{50} of 12 Torr, and a Bohr effect of - 0.48 to - 0.51. Crayfish acclimated at 9.5°C had similar P_{50} and a reduced oxygen capacity compared to crayfish kept at 15°C. There was no difference in the pre and post branchial pH of the crayfish settled in water and settled in the air for 24 hours, and consequently no change in P_{50} or capacity between venous and arterial haemolymph.

The quantity of oxygen taken up at the gills is measured by the difference between C_aO_2 and C_vO_2, and in other decapods this may be altered by pH, [lactate], [Ca^{+2}], [urate] and [caffeine] (Bridges *et al.*, 1984; Morris and Bridges, 1986; Morris *et al.*, 1985, 1986a, 1986c, 1987; Morris, Greenaway and McMahon, 1988; Taylor and Whiteley, 1989). These modulators alter the P_{50}, and the quantity of oxygen which can be carried at any given PO_2 and this has the greatest effect on oxygen content at low PO_2 values. In some Crustacea the increase in CCO_2 during aerial respiration in excess of that expected from the increase in PCO_2 is from $CaCO_3$ sources in the carapace and is associated with an increase in haemolymph [Ca^{+2}].

The $C_aO_2 - C_vO_2$ difference of the crayfish settled in the water is about 4 fold the $C_aO_2 - C_vO_2$ difference of the crayfish settled in air.

Increases in both [lactate] and [Ca^{+2}] would be expected to produce a left shift to the oxygen equilibrium curve and a reduction in P_{50}, which increases the slope of the curve considerably and enhances the haemolymph oxygen content at low PO_2.

The difference in $C_aO_2 - C_vO_2$ between the crayfish settled in the water and the crayfish settled in air is likely to be a combination of:

- an increase in cardiac stroke volume, seen in the change in amplitude in impedance recordings,
- a change in the P_{50} possibly brought about by an increase in haemolymph [lactate] and [Ca^{+2}].

Considerable work has been done on the circadian and diel rhythms of Crustacea, including the feeding habits and

migration of *P. planifrons* (Devcich, 1979), control of nocturnal locomotor activity rhythm in *P. zealandicus* (Quilter, 1975, 1977), changes in the heart rate of *Procambarus clarkii* (Pollard and Larimer, 1977), the ventilatory requirement, $\dot{V}_w/\dot{M}O_2$ (Massabuau *et al.*, 1984), and extra-cellular pH (Sakakibara *et al.*, 1987) of *Astacus leptodactylus*, the effect of temperature on the scope for activity over 24 hours in *Pacifastacus leniusculus* (Rutledge and Pritchard, 1981), and the locomotory rhythms of four species of nocturnal terrestrial crabs (Palmer, 1971). The events recorded by these authors are nocturnal increases in activity, changes in resting $\dot{M}O_2$ in water, an increase in the ventilatory requirement $\dot{V}_w/\dot{M}O_2$, increases in heart, f_H, and scaphognathite, f_R, rates and an increase in the haemolymph pH of 0.1 units in the evening.

Fingerman and Lago (1957) found a 24 hour rhythm in locomotor activity and oxygen consumption in *Orconectes clypeatus* and Rice and Armitage (1974) found a daily rhythm in the metabolic rate of *Orconectes nais*, both species demonstrating peak activity during the night. In water both *Paranephrops* species have also been found to be more active at night (Quilter, 1975; Devcich, 1979), but there is no available information to indicate that this may extend to the metabolism and respiration of a crayfish out of the water.

P. zealandicus demonstrated pH_a oscillations, in which the dusk pH_a recorded at 8 pm. was 0.1 pH units more alkaline than the dawn pH_a recorded at 8 am. The crayfish were able to maintain this regulation of pH_a, while they were out of the water. Crayfish settled in water exhibited a diel fluctuation in f_R and f_H, with high f_R and f_H recorded in the evening and low f_R and f_H in the morning. At 18°C, solitary crayfish and crayfish pairs show higher levels of emersion activity at night than during the daytime.

Massabuau *et al.* (1984) found that the ventilatory requirement of *Astacus leptodactylus* was higher in the evening than in the morning. Sakakibara *et al.* (1987) argue that this was to adjust the haemolymph pH by ventilatory control of haemolymph PCO_2, and found that the haemolymph pH of *A. leptodactylus* was 0.1 units more alkaline at dusk than

at dawn. It was suggested that the alkalosis was linked to an increase in metabolism, inducing a Bohr effect when oxygen demand was increasing (Sakakibara et al., 1987).

P. zealandicus was able to maintain this regulation of pH_a while they were out of the water. The oxygen equilibrium curve for *P. zealandicus* indicates that an increase in pH of 0.1 units in the evening would reduce the P_{50} by about 2 Torr, enabling more oxygen to be carried by the haemolymph.

P. zealandicus has a gill structure which is different from the Astacoidea in the number and position of the epipodites, Table 1.2, and is similar to the Astacoidea. The filaments described in Chapter 2 are similar to *Astacus pallipes* (Fisher, 1972), *Astacus leptodactylus* (Dunel-Erb *et al.*, 1982), *Jasus novaehollandiae* (Rogers, 1982) and *Procambarus clarkii* (Burggren *et al.*, 1974).

The maintenance of the acid-base balance during aerial excursion with a low P_aCO_2 and metabolic compensation for pH changes, while maintaining a high P_aO_2, is better managed by *P. zealandicus* than other freshwater crayfish and comparable to the high shore crab *Cyclograpsus lavauxi*, Table 5.6. The outstanding feature is the control of haemolymph pH between 7.55 and 7.74. The pH control which was maintained during emersion and submergence, when a respiratory acidosis and respiratory alkalosis are expected, also produced pH oscillations of 0.1 pH units, similar to the pH shifts reported for *Astacus leptodactylus* (Sakakibara *et al.*, 1987).

The crayfish belongs to a family, the Parastacidae, which has many terrestrial members, however little is known of their respiration physiology in or out of the water, and less is known of the terrestrial behaviour of the "aquatic" species. Consequently it is not known what demands are made on their metabolism as the extent of their activities out of the water have not been measured.

A crustacean, to be successfully bimodal, should have the ability to maintain a high haemolymph P_aO_2 and low P_aCO_2 when using aerial respiration, and be able to minimise the respiratory acidosis which accompanies the move to air, and

respiratory alkalosis which accompanies the return to the water. Conditions which enable respiration in air to be maintained without serious compromise to $\dot{M}O_2$, would also minimise the increase in [lactate] which is often part of emersion.

Does the crayfish *Paranephrops zealandicus* have a respiratory system which can cope with aerial respiration? The answer would have to be a qualified yes, as only the changes experienced by settled animals were analyzed. However two things do stand out: the animal is well able to cope with the haemolymph changes which occur when it leaves the water, and many of the activities and changes reported in this study have a diel rhythm.

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The race is run,
The job is done,
My thesis is completed,
Another week of writing
and I may have been defeated.

GLOSSARY.

This list includes uncommon abbreviations. Source of roots, Greek - (G), Latin - (L), from - f.

allopatry - *all* (G) other - *pater, patris* (L) father; in biology, from another region, with no overlapping territory.

apomorphic - *apo* (G) from, off from - *morph* (G) form, structure; the most derived form, furthest from the ancestral form.

biramous - *bi* (L) double, twice - *ramus* (L) branch; in biology, crustacean limb which has two distinct branches.

bradycardia - *brady* (G) slow - *kardia* (G) heart; a reduction in heart beat.

circadian - *circa* (L) about, approximately - *dies* (L) day; occurring or recurring about once a day, not necessarily a 24 hour cycle.

diel - *dies* (L) day; daily.

epigean - *epi* (G) on, near to, above, in addition - *ge* (G) earth; growing or living on or close to the ground.

epiphreatic - *epi* (G) on, near to, above, in addition *(phreatic)*; the zone of intermittent saturation.

euryhaline - *euros* (G) wide, *hal* (G) salt; tolerating a wide range of salinity.

hypercapnia - *huper* (G) above, over, excessive - *kapnos* (G) smoke; abnormally high concentration of carbon-dioxide in the blood.

hypogean - *hypogeus* (L), *hupogeios* (G) underground; occurring or growing beneath the surface of the earth.

hypoxia - *hupo* (G) under, lower, deficient - oxygen, late 18cent. French; medical, insufficient oxygen reaching the tissues; ecological/environmental, reduced oxygen availability.

lentic - *lenitas* (L) mildness; of freshwater organisms, inhabiting or situated in still water.

lotic - *lotus* (L) washing; of freshwater organisms, inhabiting or situated in rapidly moving water.

mybp - million years before (the) present.

phreatic - *phrear, phreat-* (G) well, cistern; pertaining to or designating water below the water table, esp. that which is capable of movement, zone of permanent

saturation.

plesiomorphic - *plesio* (G) near - *morph* (G) form, structure; the most primitive form, the nearest to the original ancestral form.

stenohaline - *stenos* (G) narrow, *hal* (G) salt; tolerating a narrow range of salinity.

sympatry - *sym* (G) together - *pater, patris* (L) father; in biology, from the same region, with common territory.

tachycardia - *tachy* (G) swift, speed - *kardia* (G) heart; an increase in heart rate, abnormally rapid heart beat.

troglo - *trogle* (G) hole; **bio** (G) life; chiefly zoological, troglobion - bia, an animal which lives exclusively in dark caves or underground caverns; troglobiotic, troglobitic - adjs; **troglophil(e)** an animal living commonly but not exclusively in caves; **trogloxene** an animal which habitually visits caves.

troglodyte - *troglodyta* (L) f. (G) *troglodutes;* an ancient Ethiopian people inhabiting caves or dens, cave dwellers, persons with primitive attitudes, an animal which lives in caves.

vadose - *vadum, vadosus* (L) shallow piece of water; pertaining to or designating underground water above the water table, the zone of aeration, the region above the level of water table fluctuations.

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APPENDIX A

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A RESPIROMETER OF FLEXIBLE DESIGN

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Abstract—1. This paper describes the construction of a respirometer used for alternate aquatic and aerial respirometry without disturbing the experimental animal within.
2. The design can accommodate a variety of chamber sizes and shapes and has excellent medium mixing properties.

INTRODUCTION

Experimental measurement of the physiological parameters of an animal is based on several assumptions. Besides the requirement for the measuring process to be accurate, the sample taken is expected to be a well mixed or representative sample and this is expected to reflect the desired state of inactivity or stress of the experimental animal.

In crustacean respirometry measuring the oxygen consumption of an animal has been approached in several ways. A mask fitted to the animal has been used to measure both branchial flow and oxygen consumption (Larimer, 1961; Arudpragasam and Naylor, 1964; Hughes *et al.*, 1969; Taylor, 1976; McMahon *et al.*, 1979; Greenaway *et al.*, 1983; O'Mahoney and Full, 1984), but this method suffers because of the tendency of many crustacea to reverse ventilation and the influence on the animal of stress from wearing the mask.

Measuring oxygen consumption from a continuous water flow through a respirometer (Taylor *et al.*, 1977a, 1977b; Butler *et al.*, 1978; Taylor and Wheatly, 1979, 1980, 1981; Wheatly and Taylor, 1981; Swain *et al.*, 1987; Wheatly, 1989) requires more equipment with more calibration.

Closed system respirometry (Sutcliffe *et al.*, 1975; Sutcliffe and Carrick, 1975; Bridges and Brand, 1980; Houlihan *et al.*, 1984; Houlihan and Innes, 1984 and Innes *et al.*, 1986) although the simplest method requires that the water within the respirometer is thoroughly mixed.

If the desired experimental protocol requires that the animal be changed from an aquatic to an aerial medium without handling and with minimal disturbance then the method must be able to accommodate both aquatic and aerial oxygen consumption measuring methods.

The need for several respirometers to measure both aquatic and aerial oxygen consumption of minimally disturbed freshwater crayfish ranging in size from several grams to 200 g led to a re-assessment of respirometer design. Closed system respirometry was preferred for simplicity. In this method the measured fall in pO_2 over time is used to calculate the oxygen consumption. With a quiescent animal accuracy is dependent on good mixing of the medium within the respirometer. Experience with available respirometers in which the medium was mixed with a magnetic "flea" suggested that the medium was very poorly mixed. This was confirmed with the use of a dye.

The respirometer design criteria were:

(1) a chamber of simple construction.
(2) Exchangeable chambers to optimize animal: medium volume ratio.
(3) Efficient and fast mixing of the aquatic medium.
(4) Simple sampling ports.
(5) Electrically safe in a wet environment.
(6) Convert from aquatic to aerial respirometry with minium disturbance to the experimental animal.

The chamber which was found to meet these requirements is illustrated in Figs 1 and 2. In essence it is a long open-ended chamber clamped between two end-plates. The end-plates have ports for water exchange with an external source when the respirometer is in the open condition. One end-plate also has an attached motor-gearbox unit driving a propeller. The chamber has additional ports for draining, sampling and inserting CO_2 absorbing materials.

MATERIALS AND METHODS

Construction

The chamber is made of 50 mm diameter perspex tube (Pt), 190 mm long with a 2.5 mm thick wall. Three holes (H1–H3) of 11 and 23 mm are bored in the tube. The size of the holes is determined by the rubber bungs being used, refer to Fig. 1. These are for aquatic medium sampling via a Luer-lock valve and syringe or connecting to a manometer for aerial respirometry (H1), supporting the fluid replacement reservoir or to allow the introduction of a container of CO_2 absorbing material (H2) and draining water from the chamber for aerial respirometry (H3).

The chamber is divided by a piece of 1 mm thick polycarbonate (F), with the upturned end glued to a section of small diameter (35 mm) perspex tube. This, the effective floor of the "animal chamber" improves the efficiency of the propeller (P) and ensures rapid circulation and mixing of the water. The size of the chamber, ie, cross-section and length, is limited only by the area of the end-plates (E1 and E2) and the length of the threaded 8 mm diameter rod (T) between the end-plates.

The end plates (E1 and E2) are of perspex sheet 10 × 80 × 120 mm to which is glued an 80 × 80 mm sheet of 3 mm thick white food-quality oil and solvent resistant Nitrile (R). One end plate (E2) has holes for the two

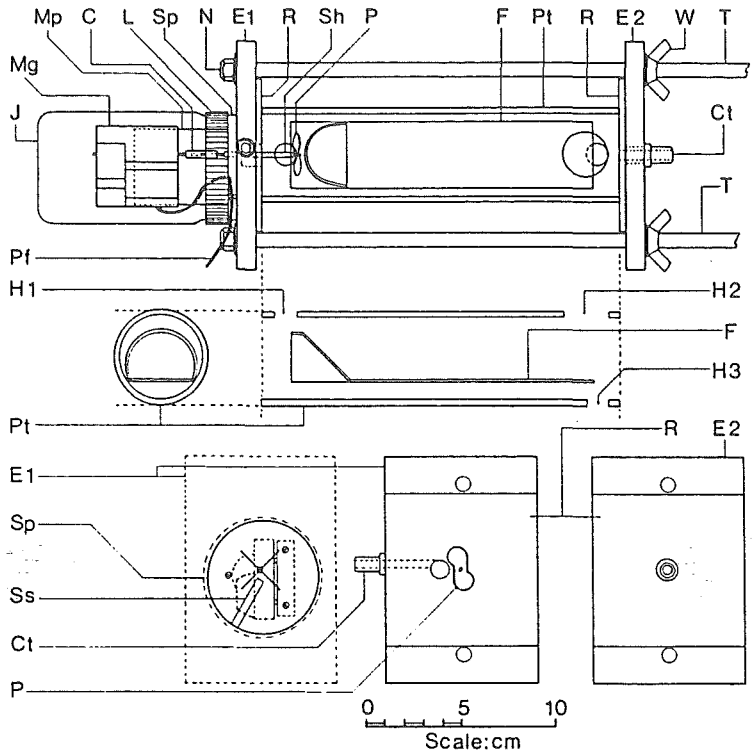

Fig. 1. Plan of the respirometer. Top, a fully assembled unit, top view, Centre, cross-section and longitudinal section of the respirometer tube, Bottom, views of the end plates, E1, left and centre, E2, right. The following key applies to the plan: (C) coupling; (Ct) connecting tube; (E1) end-plate 1; (E2) end-plate 2; (F) floor; (H1) hole 1; (H2) hole 2; (H3) hole 3; (J) jar; (L) lid; (Mg) motor and gearbox; (Mp) mounting plate; (N) "Nylock" self locking nut; (P) propeller; (Pf) power flex; (Pt) perspex tube; (R) rubber sheet; (Sh) shaft; (Sp) spacer; (Ss) spacer slot; (T) threaded rod 8 mm; (W) wingnut.

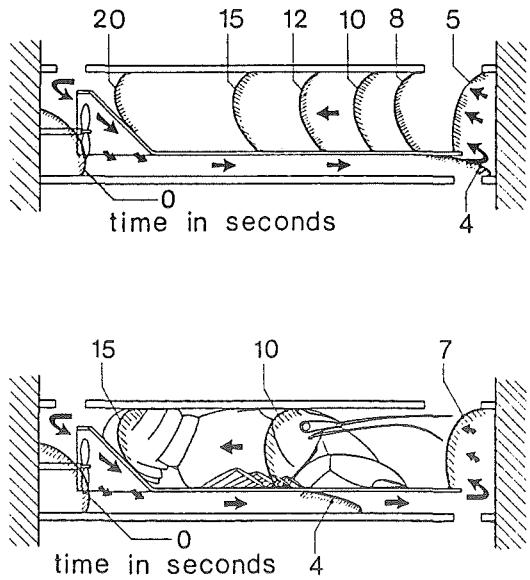

Fig. 2. Sketches made from successive photographs of the movement of dye through the respirometer. The time is in seconds, and the lines indicate the advance of the dye in that time. All three ports in the respirometer were closed with rubber bungs and the dye was introduced with an hypodermic syringe located in the bung in the left-hand port. In the top diagram the respirometer is empty and in the bottom diagram the respirometer contains a 49 g freshwater crayfish.

threaded rods (T), and a central hose connecting tube (Ct) for circulating water.

The other end plate (E1) also has two holes for the threaded rods and a centrally located propeller (P). A connecting tube (Ct) is located in the top edge of this plate and feeds the respirometer through a 4 mm diameter hole and an offset 10 mm opening. The offset causes swirling of the incoming water and ensures good mixing through the respirometer. (Because of this constant flow respirometry may be possible without the propeller and respirometer floor.)

Small electric motors suffer from two drawbacks, (1) very high speeds, and (2) high starting voltage to overcome stalling load. A toy with an appropriate motor/gearbox combination solved this problem. The motor-gearbox from an "Optima Mid Junior—1/32 scale 4WD Series" kitset four-wheel-drive off-road racer made by Tokyo Marui Co. Ltd., Japan, was used. The motor-gearbox unit is attached to the endplate (E1) and the output shaft which would have had wheels attached is coupled (C) to the propeller shaft by a piece of small bore (> 1 mm) silastic tubing. The motor rated for 3 v is operated with a single 1.5 v dry-cell.

The motor is located inside a sturdy polycarbonate screwtop jar (J) which does not leak or become distorted when the lid (L) is closed firmly. The lid with a spacer (Sp) is attached to the end plate (E1) with three screws, of which two hold the mounting plate (Mp) on which the motor-gearbox unit is mounted. All this is orientated so that the output from the motor-gearbox unit passes through the centre of the end plate.

The jar lid is separated from the end plate by a perspex spacer (Sp), a disc with a slot (Ss) for carrying the power-flex through the lid to the electric motor. The disc is firmly glued to the end plate. With the flex in place the slot carrying the

power-flex and the space between the disc and lid are filled with a single component room temperature vulcanising silicone rubber and then secured firmly with the screws.

The propeller shaft and blades are brazing rod and 1 mm brass sheet respectively and the shaft rides inside a matching piece of cannula tubing. No special materials are required as only low loads and low speeds are experienced. The cannula tube may be packed with vacuum grease to improve the seal.

The ends of the respirometer tube and the holes H1–H3 are ground smooth with a 600 grain "wet and dry" sandpaper. The holes are stopped with rubber bungs which contain, where appropriate, large bore hypodermic needles with Luer-lock three-way valves for sampling and fluid replacement.

Trials

This respirometer had a volume of 270 ml. Dye was introduced into the respirometer with a syringe and the mixing motor and a clock were started simultaneously. A sequence of photographs was taken to record the movement of the dye through the respirometer.

With a 49 g freshwater crayfish in the respirometer and a water volume of 224 ml a second sequence of photographs was taken to record the movement of dye.

RESULTS

Figure 2 shows the rate of dye movement through the respirometer. Total mixing in the empty respirometer occurred within 30 sec. The resistance to flow from the 49 g crayfish reduced the initial acceleration of the water. However, by 15 sec the water had travelled as far as it had travelled in an empty respirometer in 20 sec. This is expected as there is less water to move. With a crayfish in the respirometer total mixing was well within 30 sec.

DISCUSSION

The respirometer described in this paper has a chamber of circular section. There is no reason why a rectangular or square section would not work, except that the seal against the end-plates may not be maintained as well in the corners. A circular tube has the advantage that the ends can be machined in a lathe. A circular section also reduces the amount of dead space within the volume and ensures better mixing.

The mixing time of less than 30 sec compares well with the time of 5 min mentioned in Sutcliffe *et al.* (1975). Other authors are less specific about mixing time insisting that the stirrer prevented stratification, (Bridges and Brand 1980) or that there was adequate mixing (Wheatly, 1989). In Houlihan and Innes (1984) the water in the respirometer was thoroughly stirred with a magnetic flea and in Houlihan *et al.* (1984) the respirometer was fitted with a magnetic stirrer and the results were only included if animals showed no motor activity during the course of the measurements.

Without the floor (F) in the respirometer tube the propeller was able to mix the dye up to half way along the tube in 10 min. The dye was being circulated in a cloud about the propeller and was moving through the respirometer slowly by minor eddies.

It has been possible to match the chamber size to the animal size range, and record changes in pO_2 ranging from 2.5 torr per min for 40–60 g crayfish down to 0.35 torr per min for a 9 g crayfish in the same chamber. Chambers to accommodate larger animals up to 200 g are currently under construction.

As oxygen meters and recorders can now be used independent of mains power the overall size, weight and flexibility of this unit makes it possible to take respirometry out into the field and use a river or lake as the water bath.

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APPENDIX B

Oxygen is carried by haemolymph and blood using two mechanisms, each with different characteristics.

1) Oxygen is dissolved in plasma in a linear relationship dependent on PO_2 (Figure B.1), $C_{PLASMA} = \alpha_{PLASMA}O_2 \times PO_2$, (content = solubility x O_2 partial pressure).

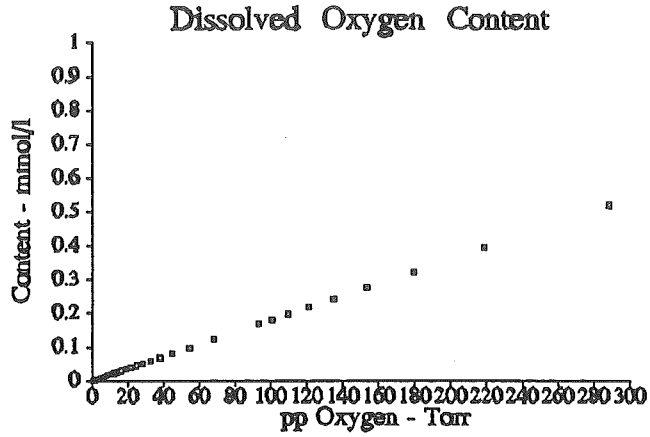

Figure B.1 the linear relationship between the oxygen partial pressure and the content dissolved in plasma.

2) Oxygen is bound to a carrier protein in the general reaction: $nO_2 + H_{cy}n \leftrightharpoons H_{cy}nO_2n$. The proportions of $H_{cy}O$ and H_{cy} depend on the PO_2. This alters the oxygen content in a nonlinear relationship (Figure B.2).

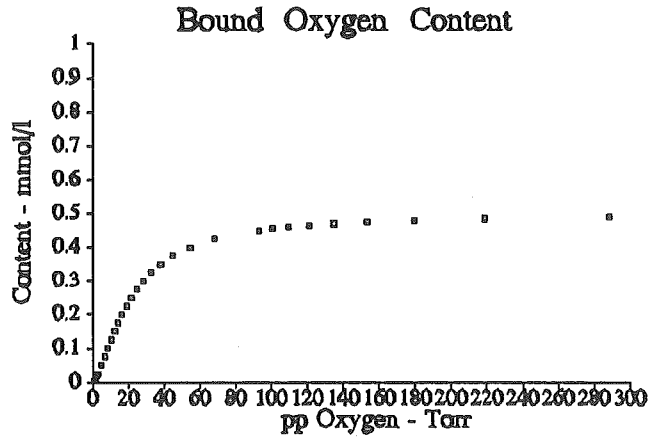

Figure B.2 the relationship between the oxygen partial pressure and the content bound to a carrier in the plasma.

The oxygen content is the contribution from both processes. This produces a composite curve (Figure B.3). As H_{cy} becomes saturated the shape of the curve is dominated by the linear relationship of physical solution.

Hill (1910) found the general equation $y = 100(kx^n / 1+kx^n)$ satisfied experimental observations from a variety of authors experimenting under a variety of conditions.

y = % H_{cy} oxygenated
k = a constant
x = PO_2

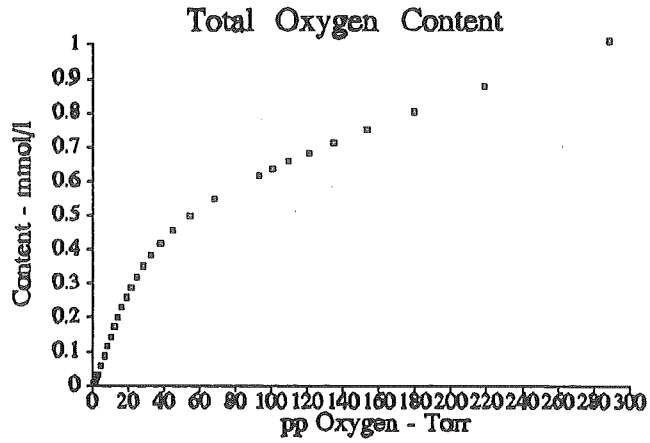

Figure B.3 the relationship between the oxygen partial pressure and the total content of oxygen, both dissolved and bound to a carrier in the plasma.

Table B.1 table of values and calculations used to generate the curves in Figure B.4 on the next page. The formulae used, and their derivation is discussed in subsequent pages. The spreadsheet Planperfect 5.1 was used for the calculations and to generate Figures B.1 to B.4.

Oxygen Equilibrium Curve

Using the equation $k(PO2)^n = (\%S/(100-\%S))$
Where %S is % saturated
k = 0.01
n = 1.5
$\alpha PlasmaO_2$ = 1.8 µmol/l/Torr
Max. bound = 500 µmol/l O_2 as HcyO

HbO%	Hb%	HbO/Hb	PO_2 Torr	Dissolved O_2 Content µmol/l	Bound O_2 Content µmol/l	Total O_2 Content µmol/l
1	99	0.0101	1.0067	1.81	5	6.8121
2	98	0.0204	1.6089	2.9	10	12.8961
3	97	0.0309	2.1228	3.82	15	18.821
4	96	0.0417	2.5894	4.66	20	24.6608
5	95	0.0526	3.0257	5.45	25	30.4463
10	90	0.1111	4.9793	8.96	50	58.9628
15	85	0.1765	6.7782	12.2	75	87.2008
20	80	0.25	8.5499	15.39	100	115.3898
25	75	0.3333	10.3574	18.64	125	143.6434
30	70	0.4286	12.2466	22.04	150	172.0439
35	65	0.5385	14.2595	25.67	175	200.667
40	60	0.6667	16.4414	29.59	200	229.5945
45	55	0.8182	18.8466	33.92	225	258.9239
50	50	1	21.5443	38.78	250	288.7798
55	45	1.2222	24.6282	44.33	275	319.3308
60	40	1.5	28.2311	50.82	300	350.8159
65	35	1.8571	32.5509	58.59	325	383.5917
70	30	2.3333	37.901	68.22	350	418.2219
75	25	3	44.814	80.67	375	455.6653
80	20	4	54.2884	97.72	400	497.719
85	15	5.6667	68.4781	123.26	425	548.2606
90	10	9	93.217	167.79	450	617.7906
91	9	10.1111	100.7394	181.33	455	636.3309
92	8	11.5	109.7653	197.58	460	657.5776
93	7	13.2857	120.8528	217.53	465	682.535
94	6	15.6667	134.8914	242.8	470	712.8046
95	5	19	153.4037	276.13	475	751.1266
96	4	24	179.2562	322.66	480	802.6611
97	3	32.3333	218.6588	393.59	485	878.5859
98	2	49	288.49	519.28	490	1009.2819
99	1	99	461.0593	829.91	495	1324.9068
99.5	0.5	199	734.3482	1321.83	497.5	1819.3268
99.9	0.1	999	2152.9982	3875.4	499.5	4374.8967
99.99	0.01	9999	9999.3333	17998.8	499.95	18498.75

In the general equation, where H is H_b or H_{cy}

$$nO_2 + H_n \leftrightharpoons H_nO_{2n} \qquad \{ 1 \}$$

1) we do not know the value of "n", which reflects the aggregation of the H units (Rutledge 1981),

2) we do know that the total amount of H is fixed, so that if the proportion of H_n in H_nO_{2n} is P, then the proportion

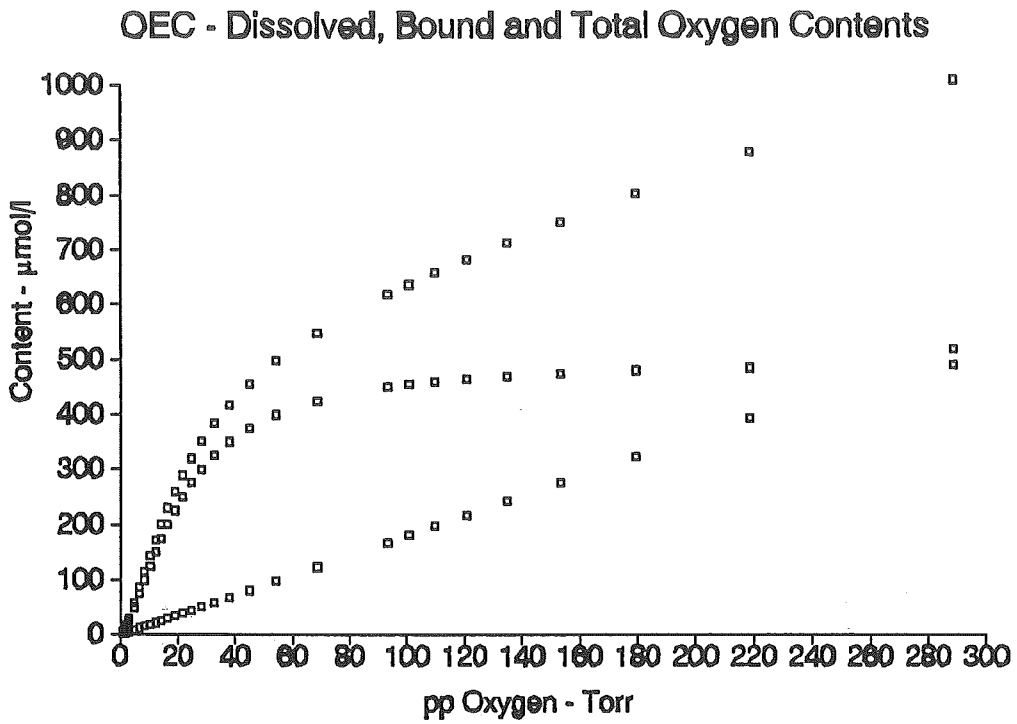

Figure B.4 plots of dissolved oxygen, bound oxygen, and total oxygen, derived from the model oxygen equilibrium curve calculated in Table B.1.

left as native H_n is (1-P), or if the amount of H_n in H_nO_{2n} is %S, the amount of unbound H_n is (100-%S).

From stoichiometry, the reaction equilibrium constant K for the equation { 1 } is given by:

$$K = \frac{[H_nO_{2n}]}{[O_2]^n[H_n]}$$

$$\Longrightarrow \quad K[O_2]^n = \frac{[H_nO_{2n}]}{[H_n]} = \frac{\%S}{100 - \%S} = \frac{P}{1 - P}$$

and $[O_2] = \alpha_{PLASMA}O_2 \times PO_2$

$$\Longrightarrow \quad K[\alpha_{PLASMA}O_2 \times PO_2]^n = \frac{P}{1 - P}$$

$$\Longrightarrow \quad K \times (\alpha_{PLASMA}O_2)^n \times (PO_2)^n = \frac{P}{1 - P}$$

if "k" is defined as $K \times (\alpha_{PLASMA}O_2)^n$ { 2 }

then $k \times (PO_2)^n = \frac{P}{1 - P}$ { 3 }

which is another form of the Hill equation.

When **P** is large (1 > P > 0), a small change in **P** produces a large change in **P/(1-P)** and a correspondingly large change in $k(PO_2)^n$, see { 3 }, and the graph of **P/(1-P)** Vs PO_2 (the partial pressure of oxygen) becomes nearly horizontal. At this point any slope in the oxygen equilibrium curve can be largely attributed to $\alpha_{PLASMA}O_2 \times PO_2$ and hence from the slope of the oxygen equilibrium curve at high values of PO_2 (> 100 Torr) a reasonable estimate for $\alpha_{PLASMA}O_2$ can be calculated. The rate of change of the curve varies with **k**, **n** and $\alpha_{PLASMA}O_2$, and the estimate for $\alpha_{PLASMA}O_2$ improves as PO_2 increases.

A PO_2 change from 100 Torr to 153 Torr, in the model in Table B.1, produced a change of 95 µmol/l in the dissolved content, and only a change of 20 µmol/l in the bound content.

From { 3 } get the linear equation -

$$log k + n log PO_2 = log(P/(1 - P)) \qquad \{ 4 \}$$

which is the Hill plot, a graph of $logPO_2$ (x axis) Vs $log(P/(1 - P))$ (y axis), which can be used to find **n**, the slope, and $logk$, the intercept on the y axis.

Having established **n** and **k** from the Hill plot, it is possible to find P_{50}, when 50% of the haemocyanin is oxygenated -

P_{50} occurs when P/1-P = 0.5/0.5 = 1
$\Longrightarrow$ $k(PO_2)^n = k(P_{50})^n = 1$, see { 3 }
and from $log k + n log P_{50} = log 1$

$$P_{50} = 10^{(log 1 - log k)/n} \qquad \{ 5 \}$$

To find the **%S**, (% saturated), or **P**, (proportion oxygenated), from the value for (P/(1-P)) or $k(PO_2)^n$, (see { 3 }), :

$$\%S = 100k(PO_2)^n / (1 + k(PO_2)^n) \qquad \{ 6 \}$$

$$P = k(PO_2)^n / (1 + k(PO_2)^n)$$

At any experimental point -

$$\text{Total oxygen content} = \alpha_{PLASMA}O_2 \times PO_2 + H_{cy}O \qquad \{ 7 \}$$

At the highest experimental PO_2, it is possible to calculate the corresponding **P** or **%S** using { 6 } which is the **max.P** or the **max.%S**, and from { 7 } it is possible to calculate the **max.$H_{cy}O$** at this experimental PO_2. **Note max.%S does not imply 100% saturated, hence the need to calculate the max.$H_{cy}O$.**

$$P_{50} \text{ content} = P_{50} \times \alpha_{PLASMA}O_2 + \text{max.}H_{cy}O \times (50/\text{max.}\%S) \quad \text{or}$$

$$= P_{50} \times \alpha_{PLASMA}O_2 + \text{max.}H_{cy}O \times (0.5/\text{max.}P) \qquad \{ 8 \}$$

It is also possible to calculate the dissociation constant K from $k = K(\alpha_{PLASMA}O_2)^n$ { 2 }. Since the value $\alpha_{PLASMA}O_2$ is temperature dependent, when the temperature changes **k** or K or both must change.

APPENDIX C

I. THE HENDERSON-HASSELBALCH EQUATION.

The reaction $AH \leftrightharpoons A^- + H^+$ has an equilibrium in which the ratio of the concentrations of $AH : A^- \times H^+$ is constant for a given set of conditions of temperature and ionic strength of the solution and can be expressed by the mass-action law (Davenport p.40),

$$[AH] : [A^-][H^+] = \text{constant}$$

or $$\frac{[A^-][H^+]}{[AH]} = K$$

The concentration of H^+, ie $[H^+]$ is measured as "pH" where by definition $pH = -\log[H^+]$, a measure of acidity/alkalinity.

and from $$\frac{[A^-][H^+]}{[AH]} = K$$

$$\log[A^-] + \log[H^+] - \log[AH] = \log K$$

$$-\log K + \log[A^-] - \log[AH] = -\log[H^+]$$

We define pK as $-\log K$ in the same way that pH is $-\log[H^+]$ and get $pK + \log[A^-] - \log[AH] = pH$

$$\Rightarrow \quad pH = pK + \log \frac{[A^-]}{[AH]}$$

which is the **Henderson - Hasselbalch** equation.

II. DISSOLUTION OF CO_2 IN H_2O

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons H^+ + HCO_3^- \leftrightharpoons 2H^+ + CO_3^{-2}$$

In three steps:

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \qquad \{1\}$$

$$H_2CO_3 \leftrightharpoons H^+ + HCO_3^- \qquad \{2\}$$

$$HCO_3^- \leftrightharpoons H^+ + CO_3^{-2} \qquad \{3\}$$

In { 1 } H_2CO_3 < 0.5%, ie more than 99.5% of the CO_2 is dissolved as molecular CO_2 (Truchot p.434). The K for { 1 } above ($[H_2CO_3]/[CO_{2(aq)}]$) is 0.00258 at 25°C, ie 0.258% at 25°C (Chang p.508).

NOTE: (Chang p.508)

$$CO_{2\,(aq)} + H_2O_{(l)} \leftrightharpoons H^+_{(aq)} + HCO_3^-{}_{(aq)}$$

or

$$H_2CO_{3\,(aq)} \leftrightharpoons H^+_{(aq)} + HCO_3^-{}_{(aq)}$$

It makes no difference whether the first or second equation is used to represent the first dissociation of carbonic acid as long as the total concentration of CO_2 in water (both hydrated and unhydrated forms) is used as the acid concentration.

Hence $[\, H_2CO_{3\,(aq)} + CO_{2\,(aq)} \,] = \alpha CO_2 \times PCO_2$ { 4 }

where PCO_2 is the partial pressure of CO_2 in Torr and αCO_2 is the solubility coefficient of CO_2 in $mmol.litre^{-1}.Torr^{-1}$

Note:- αCO_2 only describes physical solubility (Dejours p.24), not capacitance ß which for CO_2 in blood would include HCO_3^-, CO_3^{-2} and carbamino compounds.

In the solution we have the molecules of

- dissolved molecular CO_2,
- hydrated $CO_2 \rightarrow H_2CO_3$,
- dissociated $H_2CO_3 \rightarrow H^+ + HCO_3^-$,
- dissociated $HCO_3^- \rightarrow H^+ + CO_3^{-2}$.

and need to derive expressions for:-

- $[CO_2]$
- $[H_2CO_3]$
- $[HCO_3^-]$
- $[CO_3^{-2}]$

An expression for total dissolved CO_2 is written as

$$CCO_2 = CO_{2(aq)} + H_2CO_{3(aq)} + HCO_3^-{}_{(aq)} + CO_3^{-2}{}_{(aq)} \quad \{ 5a \}$$

and from equation { 4 } can substitute and get

$$CCO_2 = \alpha CO_2 \times PCO_2 + HCO_3^-{}_{(aq)} + CO_3^{-2}{}_{(aq)} \quad \{ 5b \}$$

Need expressions for $[HCO_3^-]$ & $[CO_3^{-2}]$ using the known parameters αCO_2, PCO_2, $[H^+]$, pK1 and pK2, (dissociation constants for equations { 2 } and { 3 } above), and an

expression for $[HCO_3^-] + [CO_3^{-2}]$ **Equivalents** for a Davenport diagram.

From the Henderson - Hasselbalch equations (Dejours p.57, Truchot p.434)

$$pH = pK1 + \log \frac{[HCO_3^-]}{[CO_2]} = pK1 + \log \frac{[HCO_3^-]}{[\alpha CO_2 \times PCO_2]}$$

$$pH = pK2 + \log \frac{[CO_3^{-2}]}{[HCO_3^-]}$$

With pK1 and pK2 the dissociation constants for equations { 2 } and { 3 } respectively.

$$pH - pK1 = \log[HCO_3^-] - \log[CO_2]$$

$$-\log[HCO_3^-] = -\log[CO_2] - (pH - pK1)$$

$$\log[HCO_3^-] = \log[CO_2] + (pH - pK1)$$

$$\Rightarrow [HCO_3^-] = [CO_2] \times 10^{(pH - pK1)} \quad \{ 6 \}$$

Also

$$pH - pK2 = \log[CO_3^{-2}] - \log[HCO_3^-]$$

$$-\log[CO_3^{-2}] = -\log[HCO_3^-] - (pH - pK2)$$

$$\log[CO_3^{-2}] = \log[HCO_3^-] + (pH - pK2)$$

$$\Rightarrow [CO_3^{-2}] = [HCO_3^-] \times 10^{(pH - pK2)} \quad \{ 7 \}$$

Substitute { 6 } for $[HCO_3^-]$ into { 7 } and get

$$[CO_3^{-2}] = [CO_2] \times 10^{(pH - pK1)} \times 10^{(pH - pK2)} \quad \{ 8 \}$$

Substitute { 4 }, { 6 } and { 8 } into { 5a } and get

$$CCO_2 = [CO_2 + H_2CO_3] + [HCO_3^-] + [CO_3^{-2}]$$

$$= \alpha CO_2 \times PCO_2$$

$$+ \alpha CO_2 \times PCO_2 \times 10^{(pH - pK1)}$$

$$+ \alpha CO_2 \times PCO_2 \times 10^{(pH - pK1)} \times 10^{(pH - pK2)}$$

$$= \alpha CO_2 \times PCO_2 \, (1 + 10^{(pH - pK1)} + 10^{(pH - pK1)} \times 10^{(pH - pK2)})$$

$$CCO_2 = \alpha CO_2 \times PCO_2 \ (1 + 10^{(pH - pK1)} \ (1 + 10^{(pH - pK2)})) \quad \{ 9 \}$$

And

$$PCO_2 = \frac{CCO_2}{\alpha CO_2 \ (1 + 10^{(pH - pK1)} \ (1 + 10^{(pH - pK2)}))} \quad \{ 10 \}$$

III. EQUIVALENTS FOR A DAVENPORT DIAGRAM.

An **Equivalent** is the amount of a substance that gains or loses **one mole** of electrons in a redox reaction (Chang p.345).

Thus the equivalent for HCO_3^- = $[HCO_3^-]$

and for CO_3^{-2} = $0.5 \times [CO_3^{-2}]$

If PCO_2 is measured as pH by a CO_2 permeable membrane over a pH electrode, then the electrolyte reaction in the CO_2 electrode,

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons H^+ + HCO_3^-$$

requires that all the measureables are in the form of $CO_{2(aq)}$. The acidic Cameron cell oxidises all CO_3^{-2} and HCO_3^- to $H_2CO_3 \leftrightharpoons H_2O + CO_2$, the CCO_2 being measured by the pH meter as PCO_2.

For a given pH *in vivo* the H^+ equivalents to $[HCO_3^- + CO_3^{-2}]$, where PCO_2 ($\equiv CCO_2$) and pH have been measured, is derived from equations { 6 } and { 8 }.

$$[HCO_3^- + CO_3^{-2}]Eq = [CO_2] \times 10^{(pH - pK1)} + 2 \times [CO_2] \times 10^{(pH - pK1)} \times 10^{(pH - pK2)}$$

$$= \alpha CO_2 \times PCO_2 \times 10^{(pH - pK1)} \ (1 + 2 \times 10^{(pH - pK2)})$$

Chang R., (1981) Chemistry. Random House. New York. 815pp.

Davenport H.W., (1974) The ABC of acid-base chemistry (6th ed.). The university of Chicago Press. Chicago. 124pp.

Dejours P., (1981) Principles of comparative respiratory physiology (2nd ed.). Elsevier. Amsterdam. 265pp.

Truchot J.P., (1983) Regulation of acid-base balance. Chapter 8 in Mantel L.H., (ed) Internal anatomy and physiological regulation. Volume 5, The biology of crustacea. Academic Press. New York. 471pp.

APPENDIX D

PROGRAM "BENTLINE"

Modified from the BASIC program in the text of:- Yeager D.P. and Ultsch G.R., (1989) Physiological regulation and conformation: a BASIC program for the determination of critical points. *Physiol. Zool.*, **62(4)**: 888-907.

The use of two-phase regressions is also discussed in:- Nickerson D.M., Facey D.E. and Grossman G.D., (1989) Estimating physiological thresholds with continuous two-phase regression. *Physiol. Zool.*, **62(4)**: 866-887.

```
10 AAA$ = "_____" : REM .....6/11/94
20 BBB$ = "**********************************"
30 CLS : LOCATE 1,20: PRINT "* * * PROGRAM  BENTLINE * * *"
40 PRINT
50 PRINT
60 PRINT "A program to perform a one-segment and a two-segment fit by straight"
70 PRINT "     lines to a set of at least six data points."
80 PRINT
90 PRINT "The program first computes a preliminary set of statistics to allow"
100 PRINT "     users to determine if the data will fit a straight line, and if"
110 PRINT "     that line has slope which is significantly different from zero."
120 PRINT
130 PRINT "It then finds the optimal place at which to divide the data into two"
140 PRINT "     sets so that a straight line may be fitted to each set of points."
150 PRINT
160 PRINT "The program calculates and prints the equations of the lines, the"
170 PRINT "     coordinates of the point of intersection, and statistics to"
180 PRINT "     test the hypothesis that the lines have zero slope."
190 PRINT
200 PRINT "The program can also divide the sorted data into two datasets at a"
210 PRINT "     specified point, and calculate the equations of the two lines"
220 PRINT "     which fit these two datasets."
230 PRINT
240 PRINT "PRESS ANY KEY TO CONTINUE ....." : WHILE INKEY$ = "" : WEND
250 CLS : PRINT
260 PRINT
270 PRINT "The program can handle up to 500 (X,Y) data pairs."
280 PRINT
290 PRINT
300 PRINT "Input of data pairs is:-"
310 PRINT "     a) through the keyboard,"
320 PRINT "     b) as unsorted coordinate pairs  X,Y  from disk."
330 PRINT
340 PRINT
350 PRINT "Optional output is:-"
360 PRINT "     a) sorted data file to disk,"
```

```
370 PRINT "     b) a list of the sorted data,"
380 PRINT "     c) a printed copy of the regression lines and statistics."
390 PRINT
400 PRINT
410 PRINT "PRESS ANY KEY TO CONTINUE ....." : WHILE INKEY$ = "" : WEND
420 GOTO 440
430 ERASE X,Y
440 DIM X(500), Y(500)
450 BE2 = 0 : E2 = 0
460 BM1 = 0 : M1 = 0
470 BM2 = 0 : M2 = 0
480 BB1 = 0 : B1 = 0
490 BB2 = 0 : B2 = 0
500 BK = 0 : K = 0
510 BRSX = 0 : RSX = 0
520 BRSY = 0 : RSY = 0
530 BRSX2 = 0 : RSX2 = 0
540 BRSY2 = 0 : RSY2 = 0
550 BRSXY = 0 : RSXY = 0
560 BLSX = 0 : LSX = 0
570 BLSY = 0 : LSY = 0
580 BLSX2 = 0 : LSX2 = 0
590 BLSY2 = 0 : LSY2 = 0
600 BLSXY = 0 : LSXY = 0
610 SX = 0 : SY = 0
620 SX2 = 0 : SY2 = 0 : SXY = 0
630 N = 0 : R = 0 : M = 0
640 B = 0 : E = 0 : CT = 0
650 PQ = 0 : SPL = 0
660 CLS : PRINT : PRINT
670 INPUT "Print a copy of the regression statistics .... Y / N ... ", A$
680 IF A$ = "y" OR A$ = "Y" THEN P = 1 : GOTO 700
690 IF A$ <> "n" AND A$ <> "N" THEN GOTO 670
700 PRINT
710 INPUT "Input NEW data or read OLD data from disk .... N / O ... ", A$
720 IF A$ = "N" OR A$ = "n" THEN GOSUB 2880 : GOTO 750
730 IF A$ <>"O" AND A$ <> "o" THEN GOTO 700
740 GOSUB 3000
750 PRINT : PRINT "There are "; N; " data points." : PRINT
760 IF P THEN LPRINT "There are "; N; " data points."
770 PRINT "Sorting data .... please wait." : PRINT
780 GOSUB 2280
790 INPUT "Print a copy of the sorted data .... Y / N .... ",A$ : PRINT
800 IF A$ = "n" OR A$ = "N" THEN GOTO 900
810 IF A$ <> "y" AND A$ <> "Y" THEN GOTO 790
820 PRTAB = 1
830 FOR T = 1 TO N
840   LPRINT TAB(PRTAB) USING "##########.####-"; X(T);
850   LPRINT TAB(PRTAB + 17) USING "##########.####-"; Y(T);
860   IF PRTAB = 1 THEN PRTAB = 45 ELSE PRTAB = 1 : LPRINT
870 NEXT T
```

```
880 IF PRTAB = 45 THEN LPRINT
890 LPRINT "      "; AAA$
900 INPUT "Save the sorted data to a file ..... Y / N .... ", A$ : PRINT
910 IF A$ = "Y" OR A$ = "y" THEN GOSUB 3120 : GOTO 930
920 IF A$ <> "n" AND A$ <> "N" THEN GOTO 870
930 INPUT "Divide the data at a specified point ... Y / N ...", A$ : PRINT
940 IF A$ = "N" OR A$ = "n" THEN GOTO 1080
950 IF A$ <> "Y" AND A$ <> "y" THEN GOTO 930
960 PRINT : PRINT "Data is sorted on the ascending X value" : PRINT
970 INPUT "How many points (3 or more) in the first dataset  ....", SPL : PRINT
980 IF SPL < 3 THEN GOTO 970
990 IF N - SPL > 2 THEN GOTO 1060
1000 PRINT : PRINT "ERROR - Remaining dataset must have 3 points or more" : PRINT
1010 PRINT "Dataset has total of "; N; " data points" : PRINT
1020 INPUT "Continue or Abandon .... C / A .... ", A$ : PRINT
1030 IF A$ = "C" OR A$ = "c" THEN GOTO 970
1040 IF A$ <> "A" AND A$ <> "a" THEN GOTO 1020
1050 GOTO 2100
1060 IF P THEN LPRINT : LPRINT "Data split forced, datasets of "; SPL;" and "; N-SPL; " data
points"
1070 REM * * * COMPUTE FOR INITIAL FIT OF A STRAIGHT LINE * * *
1080 SX = 0 : SY = 0 : SXY = 0 : SX2 = 0 : SY2 = 0
1090 R = 0 : M = 0 : B = 0 : E = 0
1100 FOR I = 1 TO N
1110   SX = SX + X(I)
1120   SY = SY + Y(I)
1130   SX2 = SX2 + X(I)*X(I)
1140   SY2 = SY2 + Y(I)*Y(I)
1150   SXY = SXY + X(I)*Y(I)
1160 NEXT I
1170 R = (SXY - SX*SY / N) / SQR((SX2 - SX*SX / N) * (SY2 - SY*SY / N))
1180 M = (N*SXY - SX*SY) / (N*SX2 - SX*SX)
1190 B = (SY - M*SX) / N
1200 E = SY2 - 2*B*SY - 2*M*SXY + N*B*B + 2*B*M*SX + M*M*SX2
1210 PRINT : PRINT "Regression equation for a one-segment fit:" : PRINT
1220 IF P THEN LPRINT : LPRINT "Regression equation for a one-segment fit:" : LPRINT
1230 GOSUB 2170
1240 PRINT : PRINT "Statistics for a one-segment fit:" : PRINT
1250 IF P THEN LPRINT : LPRINT "Statistics for a one-segment fit:"
1260 CT = N
1270 GOSUB 2700
1280 IF N > = 6 THEN GOTO 1320
1290 PRINT "Subsequent analysis needs at least 6 data points." : PRINT
1300 IF P THEN LPRINT "Subsequent analysis needs at least 6 data points." :LPRINT
1310 GOTO 2100
1320 REM * * FIT TWO LINES USING AS FIRST DIVIDING POINT THE VALUE OF K = 3 * *
1330 PRINT : PRINT "Seeking best two-segment fit. Please wait." : PRINT
1340 LSX = X(1) + X(2) + X(3)
1350 RSX = SX - LSX
1360 LSY = Y(1) + Y(2) + Y(3)
1370 RSY = SY - LSY
```

```
1380 LSX2 = X(1)^2 + X(2)^2 + X(3)^2
1390 RSX2 = SX2 - LSX2
1400 LSY2 = Y(1)^2 + Y(2)^2 + Y(3)^2
1410 RSY2 = SY2 - LSY2
1420 LSXY = X(1) * Y(1) + X(2) * Y(2) + X(3) * Y(3)
1430 RSXY = SXY - LSXY
1440 K = 3 : REM   Fit two lines using first dividing point at K=3.
1450 GOSUB 2580 : REM Calculate first two lines
1460 GOSUB 3230 : REM Save data
1470 IF SPL = 0 THEN GOTO 1490
1480 IF SPL = K THEN GOTO 1560
1490 K = K + 1  : REM Increase K and try again
1500 GOSUB 2460 : REM Update sums
1510 GOSUB 2580 : REM Calculate next two lines
1520 IF SPL <> 0 THEN GOSUB 3230 : GOTO 1470
1530 IF BE < E THEN GOTO 1550 : REM Compare errors
1540 GOSUB 3230 : REM Save better fit
1550 IF K < N - 3 THEN GOTO 1470 : REM Until K = N - 3
1560 PRINT : PRINT "PRESS ANY KEY TO CONTINUE ....." : WHILE INKEY$ = "" : WEND
1570 CLS : PRINT
1580 PRINT "There are "; BK; " points to the left, "; N-BK; " points to the right." : PRINT
1590 IF P = 0 THEN GOTO 1650
1600 LPRINT : LPRINT "There are"; TAB(15) BK; " points to the left,"
1610 LPRINT TAB(15) N-BK; " points to the right,"
1620 LPRINT "in a best 2-segment fit."
1630 LPRINT "     ";AAA$
1640 LPRINT : LPRINT "Regression equation for line 1 is:  " : LPRINT
1650 PRINT : PRINT "Regression equation for line 1 is:  " : PRINT
1660 M = BM1
1670 B = BB1
1680 GOSUB 2170
1690 PRINT : PRINT "Statistics for the first line: " : PRINT
1700 IF P THEN LPRINT : LPRINT "Statistics for the first line: "
1710 CT = BK
1720 SX = BLSX
1730 SY = BLSY
1740 SX2 = BLSX2
1750 SY2 = BLSY2
1760 SXY = BLSXY
1770 E = BE - BE2
1780 GOSUB 2700
1790 PRINT : PRINT "PRESS ANY KEY TO CONTINUE ....." : WHILE INKEY$ = "" : WEND
1800 CLS : PRINT
1810 PRINT "Regression equation for line 2 is: " : PRINT
1820 IF P THEN LPRINT : LPRINT "Regression equation for line 2 is: " : LPRINT
1830 M = BM2
1840 B = BB2
1850 GOSUB 2170
1860 PRINT : PRINT "Statistics for second line: " : PRINT
1870 IF P THEN LPRINT : LPRINT "Statistics for second line: "
1880 CT = N - BK
```

```
1890 SX = BRSX
1900 SY = BRSY
1910 SX2 = BRSX2
1920 SY2 = BRSY2
1930 SXY = BRSXY
1940 E = BE2
1950 GOSUB 2700
1960 REM * * * CALCULATE THE POINT OF INTERSECTION * * *
1970 XI = (BB2 - BB1) / (BM1 - BM2)
1980 YI = BM1 * XI + BB1
1990 XT = (X(BK) + X(BK + 1)) / 2
2000 PRINT
2010 PRINT "The two lines intersect at  x = "; XI; "  and  y = "; YI
2020 PRINT
2030 PRINT "The transition occurs between  x = "; X(BK); "  and  x = "; X(BK+1)
2040 PRINT
2050 PRINT "The midpoint of this interval is  x = "; XT
2060 IF P = 0 THEN GOTO 2110
2070 LPRINT : LPRINT "The two lines intersect at  x = "; XI; "  and  y = "; YI
2080 LPRINT "The transition occurs between  x = "; X(BK); "  and  x = "; X(BK+1)
2090 LPRINT "The midpoint of this interval is  x = "; XT
2100 LPRINT : LPRINT "     ";BBB$ ;BBB$ : LPRINT CHR$(12)
2110 PRINT
2120 INPUT "Do you want to analyse another dataset ... Y / N .. ",A$
2130 IF A$ = "N" OR A$ = "n" THEN GOTO 2160
2140 IF A$ <> "Y" AND A$ <> "y" THEN GOTO 2100
2150 GOTO 430
2160 END
2170 REM * * * SUBROUTINE TO PRINT EQUATION * * *
2180 IF B <= 0 THEN PRINT "    Y   =   -  "; ELSE PRINT "    Y   =  ";
2190 PRINT USING "######.######"; ABS(B);
2200 IF M <= 0 THEN PRINT "  -  "; ELSE PRINT "  +  ";
2210 PRINT USING "######.######"; ABS(M); : PRINT " X"
2220 IF P = 0 THEN GOTO 2270
2230 IF B <= 0 THEN LPRINT "    Y   =   -  "; ELSE LPRINT "    Y   =  ";
2240 LPRINT USING "######.######"; ABS(B);
2250 IF M <= 0 THEN LPRINT "  -  "; ELSE LPRINT "  +  ";
2260 LPRINT USING "######.######"; ABS(M); : LPRINT " X"
2270 RETURN
2280 REM * * * SORT PROCEDURE - ALGORITHM IS BUBBLE SORT * * *
2290 I = 1
2300 IF I > N - 1 THEN GOTO 2450
2310   SW = 0
2320   FOR J = 1 TO N - I
2330     IF X(J) < X(J + 1) THEN GOTO 2420
2340     IF X(J) = X(J + 1) AND Y(J) <= Y(J + 1) THEN GOTO 2420
2350       T = X(J + 1)
2360       X(J + 1) = X(J)
2370       X(J) = T
2380       T = Y(J + 1)
2390       Y(J + 1) = Y(J)
```

```
2400        Y(J) = T
2410        SW = 1
2420    NEXT J
2430 I = I + 1
2440 IF SW <> 0 THEN GOTO 2300
2450 RETURN
2460 REM * * * SUBROUTINE TO UPDATE SUMS * * *
2470 LSX = LSX + X(K)
2480 LSY = LSY + Y(K)
2490 LSX2 = LSX2 + X(K)^2
2500 LSY2 = LSY2 + Y(K)^2
2510 LSXY = LSXY + X(K) * Y(K)
2520 RSX = RSX - X(K)
2530 RSY = RSY - Y(K)
2540 RSX2 = RSX2 - X(K)^2
2550 RSY2 = RSY2 - Y(K)^2
2560 RSXY = RSXY - X(K) * Y(K)
2570 RETURN
2580 REM * * * SUBROUTINE TO FIT TWO STRAIGHT LINES TO THE DATA * * *
2590 M1 = (K * LSXY - LSX * LSY) / (K * LSX2 - LSX*LSX) : REM Slope &
2600 B1 = (LSY - M1 * LSX) / K : REM Y-intercept &
2610 E1 = LSY2 - 2 * B1 * LSY - 2 * M1 * LSXY + K * B1 * B1 : REM Error
2620 E1 = E1 + 2 * B1 * M1 * LSX + M1 * M1 * LSX2 : REM For line 1
2630 KN = N - K
2640 M2 = (KN * RSXY - RSX * RSY) / (KN * RSX2 - RSX * RSX) : REM Slope &
2650 B2 = (RSY - M2 * RSX) / KN : REM Y-intercept &
2660 E2 = RSY2 - 2 * B2 * RSY - 2 * M2 * RSXY + KN * B2 * B2 : REM Error
2670 E2 = E2 + 2 * B2 * M2 * RSX + M2 * M2 * RSX2 : REM For line 2
2680 E = E1 + E2 : REM Sum errors of the two lines
2690 RETURN
2700 REM * * * SUBROUTINE FOR COEFFICIENT OF DETERMINATION AND t-TEST * * *
2710 DF = CT - 2
2720 MS = E / DF
2730 S2 = MS / (SX2 - SX^2/CT)
2740 S = S2^.5
2750 R = (SXY - SX * SY / N) / SQR((SX2 - SX * SX / N) * (SY2 - SY * SY / N))
2760 PRINT "  Coefficient of determination: ";R*R
2770 PRINT "  Slope: ";M
2780 PRINT "  Standard error of slope:  ";S
2790 PRINT "  t-statistic: "; M/S
2800 PRINT "  Degrees of freedom: "; DF : PRINT
2810 IF P = 0 THEN GOTO 2870
2820 LPRINT "  Coefficient of determination: ";R*R
2830 LPRINT "  Slope: ";M
2840 LPRINT "  Standard error of slope:  ";S
2850 LPRINT "  t-statistic: "; M/S
2860 LPRINT "  Degrees of freedom: "; DF : LPRINT "      ";AAA$
2870 RETURN
2880 REM * * * SUBROUTINE FOR DATA INPUT FROM THE KEYBOARD * * *
2890 PRINT
2900 PRINT "Enter data points as ordinate pairs,"
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2910 PRINT ".... ie X,Y .... one pair to a line."
2920 PRINT "Enter two zeros ... 0,0 ... to stop."
2930 I = 0
2940 I = I + 1
2950   INPUT ".... ", X(I), Y(I)
2960   IF X(I) = 0 AND Y(I) = 0 THEN GOTO 2980
2970 GOTO 2940
2980 N = I - 1
2990 RETURN
3000 REM * * * SUBROUTINE FOR DATA INPUT FROM A DISK FILE * * *
3010 PRINT : INPUT "Name the input file of data pairs ... (drive:\dir\filename)   ", F$
3020 IF P THEN LPRINT "Name of the input file for data pairs ... "; F$
3030 OPEN F$ FOR INPUT AS #1
3040 I = 0
3050 I = I + 1
3060   INPUT #1, X(I), Y(I)
3070   IF X(I) = 0 AND Y(I) = 0 THEN N = I - 1 : GOTO 3100
3080   IF EOF(1) THEN N = I : GOTO 3100
3090 GOTO 3050
3100 CLOSE #1
3110 RETURN
3120 REM * * * SUBROUTINE TO SAVE THE SORTED DATA TO A DISK FILE * * *
3130 PRINT : INPUT "Output file name ... (Drive:\dir\filename)  "; F$ : PRINT
3140 IF P THEN LPRINT : LPRINT "Output file name ... "; F$ : LPRINT
3150 IF F$= "" THEN GOTO 3220
3160   OPEN F$ FOR OUTPUT AS #2
3170   FOR I = 1 TO N
3180     PRINT #2, X(I); ", "; Y(I)
3190   NEXT I
3200   PRINT #2, 0; ", "; 0
3210   CLOSE #2
3220 RETURN
3230 REM * * * SUBROUTINE TO SAVE THE BEST RESULTS CALCULATED SO FAR * * *
3240 BE = E
3250 BE2 = E2
3260 BM1 = M1
3270 BM2 = M2
3280 BB1 = B1
3290 BB2 = B2
3300 BK = K
3310 BRSX = RSX
3320 BRSY = RSY
3330 BRSX2 = RSX2
3340 BRSY2 = RSY2
3350 BRSXY = RSXY
3360 BLSX = LSX
3370 BLSY = LSY
3380 BLSX2 = LSX2
3390 BLSY2 = LSY2
3400 BLSXY = LSXY
3410 RETURN
```